The role of MAPK signalling pathways in leukemic cell death

Thesis submitted in accordance with the requirements of the University of Chester for the degree of Doctor of Philosophy by

Michelle Cordingley

September 2018

University of Chester

Declaration

The material being presented for examination is my own work and has not been submitted for an award of this or another HEI except in minor particulars which are explicitly noted in the body of the thesis. Where research pertaining to the thesis was undertaken collaboratively, the nature and extent of my individual contribution has been made explicit.

Signed:

Date:

Acknowledgements

First and foremost, I would like to thank my supervisors, Professor John Williams and Dr Elyse Ireland, for their invaluable support and guidance throughout the project. Not only am I grateful for the opportunity to carry out a PhD, I also appreciate all of the other opportunities they have provided me with whilst studying at the University of Chester.

I would like to give special thanks to my Mum for her continuous support throughout everything, she is always there for me. I promise this is my last degree!

I am thankful to all of my family and friends for their support. Thank you to my brother Steven for his time and effort in helping me with my IT issues. A special mention to my wonderful niece and nephew, Chloe & Oliver, who always make me smile. Thank you to Mike for constantly telling me not to panic and distracting me from my PhD by taking me away to pretty countries. Stuart, Sophie, Naomi and Matt- the best friends anyone could have. Thank you for believing in me when I did not believe in myself.

A big thank you to everyone at Chester Medical School, their words of encouragement have been much appreciated. Thank you to Jasmine Stanley-Ahmed for her advice and friendship and to Hanady Hamdallah for her support. Thanks to all of the past and present members of the lab. Thank you to Taha Contractor, Sheah Lin Lee and Gareth Shaw, whose advice and guidance has been invaluable since the start of the project.

Abstract

Mitogen-activated protein kinase (MAPK) signalling pathways are important signalling pathways involved in mediating various cellular processes including both cell survival and cell death. The c-Jun N-terminal kinase (JNK) pathway, the p38 pathway and the extracellular signal-regulated kinase (ERK1/2) pathway are three well-studied conventional MAPK signalling pathways. Previous research has shown these MAPK signalling pathways play an important role in the development and progression of leukaemia and in the response of leukemic cells to therapy. Whilst it appears to be well established that the constitutive activation of ERK mediates leukemic cell survival, the roles of the JNK and p38 signalling pathways in leukemogenesis, in particular the role in acute myeloid leukaemia (AML), are less well understood.

This thesis investigates the role of the JNK, p38 and ERK signalling pathways in leukemic death. MAPK signalling pathways were targeted in the U937 monocytic cell line using small molecule MAPK inhibitors in combination with various cell stressors: UV light, chemotherapeutic agents (doxorubicin and vincristine) and heat treatment. The effects on cell death were examined using plate-based assays, flow cytometry and fluorescence microscopy. Preliminary investigations were also performed in peripheral blood mononuclear cells (PBMCs) from healthy individuals to allow a comparison to non-leukemic cells.

Results show inhibition of ERK signalling in U937 cells induced cell death and ERK signalling had little effect on UV-induced and heat treatment-induced cell death. JNK signalling and p38 signalling provided protection against UV-induced cell death in both U937 cells and in PBMCs from healthy individuals. JNK and p38 signalling mediated cell survival in response to heat treatment to a certain extent. JNK signalling was required for the induction of cell death induced by doxorubicin whereas p38 signalling provided a level of protection against doxorubicin-induced cell death. U937 cells were found to be more sensitive to vincristine treatment than PBMCs from healthy individuals and the activation of JNK and p38 signalling was essential for vincristine-induced cell death in U937 cells.

Taken together, the results presented in this thesis demonstrate that the roles of the JNK, p38 and ERK signalling pathways in leukemic cell death are stimuli-specific. This highlights the importance of understanding the involvement of particular pathways in the response to specific chemotherapeutic agents, in order to provide effective leukaemia therapy. Therapeutic inhibition of MAPK signalling pathways to increase the sensitivity of leukemic cells to chemotherapy could be beneficial when MAPKs are involved in providing protection against chemotherapy-induced cell death. For chemotherapies which require MAPK activation for cell death, failure to activate MAPKs may provide a mechanism for chemoresistance. Therapeutic methods to enhance activation of the pathways provide a possible approach to increase the susceptibility of leukemic cells to death.

Table of Contents

List of Figures

List of Tables

Abbreviations

AKT	Protein kinase B
ALL	Acute lymphocytic leukaemia
AML	Acute myeloid leukaemia
ANOVA	Analysis of variance
AP-1	Activator protein-1
APAF-1	Apoptotic protease activating factor 1
APS	Ammonium persulphate
ARA-C	Cytarabine
ASK	Apoptosis signal-regulating kinase
ATP	Adenosine triphosphate
B-ALL	B-cell acute lymphocytic leukaemia
B-CLL	B-cell chronic lymphocytic leukaemia
BSA	Bovine serum albumin
CHK1/CHK2	Checkpoint kinase 1/2
CLL	Chronic lymphocytic leukaemia
CML	Chronic myeloid leukaemia
CXCL12	Stromal-derived factor-1α
CXCR4	Chemokine receptor type 4
DAPI	4’,6-Diamidino-2-Phenylindole
dH_2O	Distilled water
DLK	Dual leucine zipper kinase
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DSBs	Double-strand breaks
DUSP	Dual-specificity phosphatase
EGF	Epidermal growth factor
ERK	Extracellular signal-regulated kinase
FAK	Focal adhesion kinase
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FTI	Farnesyltransferase inhibitors
FLT3	FMS-like tyrosine kinase 3

GDP	Guanosine diphosphate
GRB2	Growth factor receptor-bound protein 2
GTP	Guanosine triphosphate
HSPs	Heat shock proteins
HTLV-1	Human T cell leukaemia virus type 1
IGF-1	Insulin-like growth factor 1
IFN-α	Interferon-α
IL-10	Interleukin 10
JIP1	JNK-interacting protein-1
JNK	c-Jun N-terminal kinase
kDa	Kilo dalton
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MAPKAPK-2	Mitogen-activated protein kinase-activated protein kinase-2
MAPKK	Mitogen-activated protein kinase kinase
MAPKKK	Mitogen-activated protein kinase kinase kinase
MDR1	Multi-drug resistance protein
MHC	Major histocompatibility complex
MLK	Mixed lineage kinase
MMP-9	Metalloproteinase-9
NF-κB	Nuclear factor-κB
NGF	Nerve growth factor
PBS	Phosphate buffered saline
PBMCs	Peripheral blood mononuclear cells
PDGF	Platelet-derived growth factor
PES	Phenazine ethosulphate
P-GP	P-glycoprotein
PI	Propidium iodide
PI3K	Phosphoinositide 3-kinase
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
SD	Standard deviation
SDS	Sodium dodecyl sulphate

SOS	Son of Sevenless
T-ALL	T-cell acute lymphocytic leukaemia
TGF-α	Transforming growth factor alpha
TGF-β	Transforming growth factor beta
TNF-α	Tumour necrosis factor alpha
TPL2	Tumor progression locus 2
TTBS	Tris buffered saline- Tween
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
v/v	Volume by volume
w/v	Weight by volume

Chapter 1

Introduction

Chapter 1: Introduction

1.1 Rationale for the study

Cell signalling is a critical process involved in co-ordinating a range of cellular processes including: cell growth, cell differentiation, inflammation, metabolism and cell death (Uings & Farrow, 2000). Deregulation of complex signalling networks underpins the molecular basis of many diseases, including cancer. Driven by genetic changes, abnormalities in signalling pathways allow cancer cells to rapidly proliferate, survive and often invade other tissues (Giancotti, 2014; Kolch, Halasz, Granovskaya, & Kholodenko, 2015; Sever & Brugge, 2015). Understanding which signalling pathways are deregulated, and the ways in which these pathways are changed, will provide a greater insight into the mechanisms involved in carcinogenesis. It is also important to gain knowledge of how signalling pathways are altered in response to chemotherapy. Targeting signalling pathways in cancer cells is of great therapeutic value. Although there are general principles underlying all cancers, signalling pathways are altered in a tissue and cell-type specific manner (Giancotti, 2014). Therefore, in order to provide effective cancer therapy, it is necessary to understand how signalling pathways are deregulated specifically in each type of cancer.

The work presented in this thesis will focus on leukaemia, a class of haematological cancers which originate in the bone marrow and result in the production of immature or abnormal leucocytes (Figure 1.1 and Table 1.1). Of the 357,000 new cases of cancer reported in the United Kingdom (UK) in 2014, around 9,500 of these were cases of leukaemia (Cancer Research UK, 2017). In 2014, there were around 4,500 deaths in the UK because of leukaemia. These figures highlight the important requirements for improved treatments for leukaemia, with the ultimate aim of increasing survival rates. Gaining a greater understanding of how signalling pathways function in the development of leukaemia and in the response to treatment will aid future leukaemia therapy. This thesis will investigate the role of a particular class of signalling pathways, the mitogen-activated protein kinase (MAPK) signalling pathways, and the role these signalling pathways play in leukemic cell death.

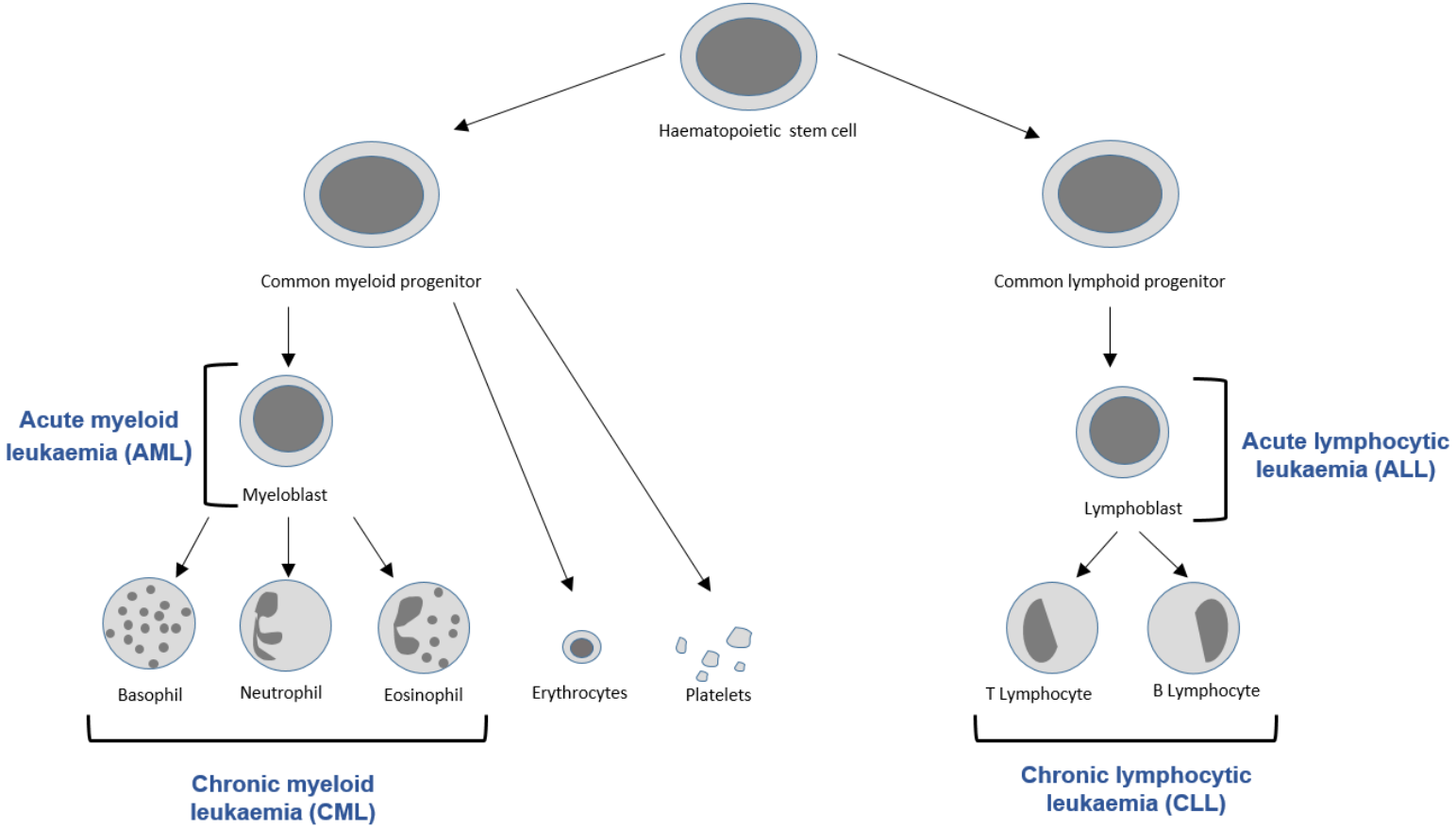

Figure 1.1: Haematopoiesis and the development of leukaemia
During haematopoiesis, haematopoietic cells are generated from stem cells in the bone marrow via the myeloid or lymphoid lineage. Defects in haematopoiesis result in diseases such as leukaemia. Acute leukaemia is characterised by a rapid increase in immature blasts whereas chronic leukaemia is characterised by the build-up of mature, abnormal leucocytes.

Table 1.1: Classification of leukaemia
(Information taken from Cancer Research UK, 2017)

Type of leukaemia	Incidence	Subtypes
Acute myeloid leukaemia (AML)	Most common type of leukaemia Highest incidence in older adults – incidence rates increase rapidly from around 40 years old More common in males (57%) than females (43%)	Myeloblastic leukaemia (M0, M1, M2) Acute promyelocytic leukaemia (M3) Acute myelomonocytic leukaemia (M4) Acute monocytic leukaemia (M5) Acute erythroid leukaemia (M6) Acute megakaryoblastic (M7)
Acute lymphocytic leukaemia (ALL)	Most common in children, teenagers and young adults- highest incidence in children less than 4 years old More common in males (59%) than females (41%)	Acute precursor B-cell leukaemia Acute B-lymphoblastic leukaemia Acute T-lymphoblastic leukaemia
Chronic myeloid leukaemia (CML)	Highest incidence in older adults – incidence rates increase rapidly from around 55 years old More common in males (59%) than females (41%)	Chronic granulocytic leukaemia (CGL) Juvenile CML Chronic neutrophilic leukaemia (CNL) Chronic myelomonocytic leukaemia (CMML) Atypical CML
Chronic lymphocytic leukaemia (CLL)	Very rare in children and young adults. Highest incidence in older adults- incidence rates increase rapidly from 45 years old More common in males (63%) than females (37%)	B-cell chronic lymphocytic leukaemia (B-CLL) T-cell chronic lymphocytic leukaemia (T-CLL) Hairy cell leukaemia

1.2 Cancer Development

The development and progression of cancer is a multi-step process driven by genetic changes within a cell. The transformation of a 'normal' non-cancer cell, into a rapidly proliferating, abnormal cancer cell is an extremely complex process, which results in a small number of important underlying properties. These general properties are described as the 'hallmarks of cancer' (Figure 1.2) (Hanahan & Weinberg, 2011; Hanahan & Weinberg, 2000).

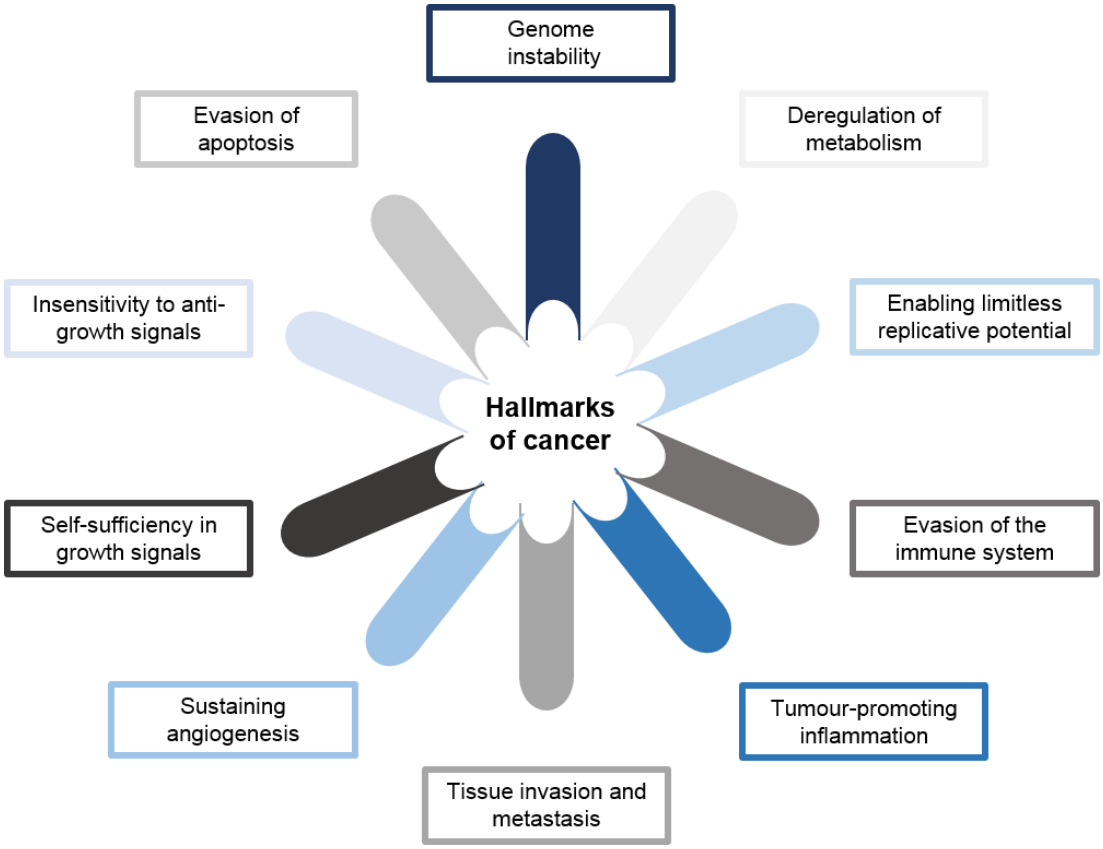

Figure 1.2: Hallmarks of cancer
Six hallmarks of cancer were initially identified: self-sufficiency in growth signals, insensitivity to anti-growth signals, evasion of apoptosis, enabling limitless replicative potential, sustaining angiogenesis, and tissue invasion and metastasis. A further four hallmarks were later proposed: evasion of the immune system, deregulation of metabolism, genome instability and tumour-promoting inflammation. (Adapted from Hanahan & Weinberg, 2011; Hanahan & Weinberg, 2000).

Deregulation of cell signalling pathways underlies each of the hallmarks of cancer (Giancotti, 2014; Sever & Brugge, 2015) and examples of signalling pathways affecting these properties will briefly be considered:

- **Genome instability** - accumulation of genetic alterations during cell division is one of the underlying principles for the development of cancer (Pikor, Thu, Vucic, & Lam, 2013). Genomic instability in cancer cells occurs mainly as a result of failure in DNA repair mechanisms and cell cycle checkpoint signalling (Shen, 2011). The ATM-Chk2-p53 and ATR-Chk1-p53 signalling pathways involved in DNA damage signalling, are often deregulated in cancer cells (Smith, Tho, Xu, & Gillespie, 2010). Inactivation of the p53 tumour suppressor, which is involved in an extensive number of signalling pathways, contributes to genome instability (Negrini, Gorgoulis, & Halazonetis, 2010).

- **Deregulation of metabolism** - metabolism in cancer cells is reprogrammed towards aerobic glycolysis (Warburg effect) in order to meet the anabolic demands required for growth and proliferation (Phan, Yeung, & Lee, 2014). Metabolic reprogramming in cancer cells is the result of mutations in oncogenes and tumour suppressors which alter signalling pathways involved in metabolism, for example the frequently hyperactivated PI3K-AKT-mTOR signalling pathway (Khan, Yap, Yan, & Cunningham, 2013; Lien, Lyssiotis, & Cantley, 2016).

- **Enabling limitless replicative potential** - cancer cells develop mechanisms which allows the cells to divide indefinitely. The main mechanism utilised by cancer cells is the reactivation of telomerase, which occurs in greater than 85% of cases of cancer (Akincilar, Unal, & Tergaonkar, 2016). Reactivation of telomerase is achieved by the transcriptional activation of telomerase reverse transcriptase (TERT) which is controlled by signalling pathways involving the transcription factors Myc, β-catenin and nuclear factor κB (NF-κB) (Li & Tergaonkar, 2016).

- **Evasion of the immune system** - cancer cells evade the immune system by three main mechanism: loss of antigenicity, loss of immunogenicity and by creation of an immunosuppressive cancer microenvironment (Beatty & Gladney, 2015). Loss of antigenicity is mainly due to defective antigen processing and presentation, such as the downregulation of pathways in cancer cells which affect the major histocompatibility complex (MHC) I pathway (Vinay et al., 2015). Loss of immunogenicity can occur by the secretion of suppressive cytokines such as interleukin-10 (IL-10) and transforming growth factor (TGF-β) and the downstream pathways affected by these cytokines.

- **Tissue invasion and metastasis** - in order for metastasis to occur, cancer cells must break away from the primary tumour, invade connective tissues and spread to distant sites in the body (Jiang et al., 2015). Loss of cell-cell adhesion, for examples through the loss of E-cadherin, allows dissociation of cancer cells from the primary site and in addition changes in cell-matrix interactions enable cells to invade connective tissue. Signalling pathways involved in maintaining cell-cell adhesion and cell-matrix interactions, such as PI3/AKT and FAK/integrin signalling pathways are altered in cancer cells (Zhao & Guan, 2011).

- **Sustaining angiogenesis** - cancer cells require a constant supply of oxygen and nutrients to survive which is facilitated by the establishment of a vascular network, termed angiogenesis. The most commonly deregulated signalling pathway which mediates cancer angiogenesis is vascular endothelial growth factor (VEGF)-receptor signalling (Nishida, Yano, Nishida, Kamura, & Kojiro, 2006; Ziyad & Iruela-Arispe, 2011).

- **Tumour promoting inflammation** - inflammation plays a critical role in the development of cancer; cancer microenvironments are often infiltrated with inflammatory cells (Coussens & Werb, 2002). NF-κB signalling is an important pathway involved in inflammation and deregulation of NF-κB signalling has been reported in many cancers (Colotta, Allavena, Sica, Garlanda, & Mantovani, 2009). NF-κB signalling in inflammatory cells can

stimulate NF-κB signalling in cancer cells, causing the recruitment of further immune cells, such as tumour-associated macrophages (TAMs) to the cancer site. TAMs support cancer development by aiding cell proliferation, invasion and metastasis, stimulating angiogenesis and inhibiting the anti-cancer immune response (Liu & Cao, 2015).

Although each of these hallmarks of cancer explains the general principles underlying the development and progression of cancer, there is variability in the extent to which they apply to specific cancers. For example, in leukaemia, sustained angiogenesis and tissue invasion and metastasis are less important due to the location of the leukemic cells in the bone marrow and blood stream. The most important 'hallmarks of cancer' in terms of the research presented in the thesis are: self-sufficiency in growth signals, insensitivity to anti-growth signals and evasion of apoptosis:

- **Self-sufficiency in growth signals** - constitutive activation of signalling pathways involved in growth is a feature of cancer cells; it enables cells to progress through the cell cycle independently of external signals. Self-sufficiency in growth signals can be achieved by multiple mechanisms. One such mechanism is the self-production of growth factors such as platelet-derived growth factor (PDGF) or transforming growth factor-α (TGF-α). An alternative mechanism is increased levels of growth factor receptors, for example the upregulation of epidermal growth factor receptors (EGFR) (Gutschner & Diederichs, 2012; Hanahan & Weinberg 2000). Self-sufficiency in growth signals can also occur as a result of constitutive activation of signalling components downstream of growth factor-receptor binding. Components of the Ras-Raf-MEK-ERK signalling pathway are frequently deregulated in many cancers (Santarpia, Lippman, & El-Naggar, 2012). Disruption of negative-feedback mechanisms can also account for self-sufficiency in growth signals (Gutschner & Diederichs, 2012).

- **Insensitivity to anti-growth signals** - in non-cancer cells, cell proliferation is tightly regulated by negative regulators which control cell division. Cancer cells can become insensitive to anti-growth signals via several

mechanisms. One mechanism is the downregulation of anti-growth factor receptors, such as the transforming growth factor-β (TGF-β) receptor. Insensitivity to anti-growth signals also occurs due to inactivation of the tumour suppressors, p53 and the retinoblastoma protein (Sherr & McCormick, 2002).

- **Evasion of apoptosis** - cancer cells acquire the ability to evade apoptosis, a form of programmed cell death. Evasion of apoptosis typically occurs due to deregulation of apoptotic machinery. This can result in either the upregulation of anti-apoptotic proteins (Bcl-2) and inhibitors of apoptosis (IAPs) or the down-regulation of pro-apoptotic proteins (Bim, Bax, Bad) (Fernald & Kurokawa, 2013). Various upstream signalling pathways which regulate apoptosis are deregulated in cancer and include the PI3-kinase/Akt signalling pathway, c-Jun N-terminal kinase pathway and p38 signalling pathway. Loss of p53 also provides cancer cells with a mechanism for apoptosis evasion (Fridman & Lowe, 2003).

1.3 Mitogen-activated protein kinase signalling

Mitogen-activated protein kinases (MAPKs) are a family of serine/threonine protein kinases which are highly conserved amongst eukaryotes (Cargnello & Roux, 2011). MAPKs, are responsible for relaying, amplifying and integrating signals from an extracellular stimuli into an intracellular response (Zhang & Liu, 2002). MAPK signalling pathways regulate a diverse range of cellular processes. These include: differentiation, proliferation, growth and apoptosis, in addition to the involvement of MAPKs in both the stress and immune responses (Plotnikov, Zehorai, Procaccia, & Seger, 2011; Wada & Penninger, 2004; Zhang, Liu, & Liu, 2002; Zhang & Dong, 2005). This section will discuss the current knowledge regarding MAPK signalling pathways, their role in leukemogenesis and in response to leukaemia therapy, and in addition, how these pathways can be targeted.

1.3.1 MAPK signalling cascade

MAPK signalling pathways are activated in response to a range of external stimuli including growth factors, cytokines, and cell stresses such as ultraviolet (UV) light, temperature, osmolarity, hypoxia and drugs (Cuevas, Abell, & Johnson, 2007; Kyriakis & Avruch, 2001). Each MAPK signalling pathway is organised into a three-tiered protein kinase cascade consisting of three protein kinases: mitogen-activated protein kinase kinase kinases (MAPKKKs), mitogen-activated protein kinase kinases (MAPKKs) and mitogen-activated protein kinases (MAPKs), which are activated sequentially (Figure 1.3). At least twenty MAPKKKS, seven MAPKKs and at least eleven MAPKs have been identified (Dhanasekaran & Johnson, 2007).

MAPKKKs, alternatively named MAP3K or MEKKK, are the first kinases in the cascade to be activated. MAPKKKs are activated in response to stimuli, via receptor-dependent and receptor-independent mechanisms, typically involving interactions with signalling proteins such as GTP-binding proteins (Munshi & Ramesh, 2013). MAPKKKs dually phosphorylate serine or threonine residues at conserved positions in the activation loop of the downstream substrate, a MAPKK, alternatively termed MAP2K or MEK. Complexity and diversity of MAPK signalling is achieved by the ability of a single MAPKK to be activated by multiple MAPKKKs (Johnson, Dohlman, & Graves, 2005).

MAPKKs are responsible for phosphorylating and activating MAPKs. All MAPKs contain a Tyr-X-Thr motif within an activation loop, located in a kinase domain between the N-terminal and C-terminal domain (Cargnello & Roux, 2011). ‘X’ is either a glutamate, proline or glycine and is dependent on the individual MAPK protein. Dual phosphorylation, by the appropriate MAPKK, of both the tyrosine and threonine residue within the motif, is required for the activation of the MAPK. MAPKs activate a range of MAPK-activated protein kinases (MAPKAPKs), which are located in various subcellular locations. In the cytoplasm, MAPKs control phosphorylation of other kinases and cytoskeletal proteins. Alternatively, MAPKs can translocate into the nucleus and phosphorylate transcription factors involved in gene expression. Collectively, these control the appropriate cellular process in response to the given stimuli.

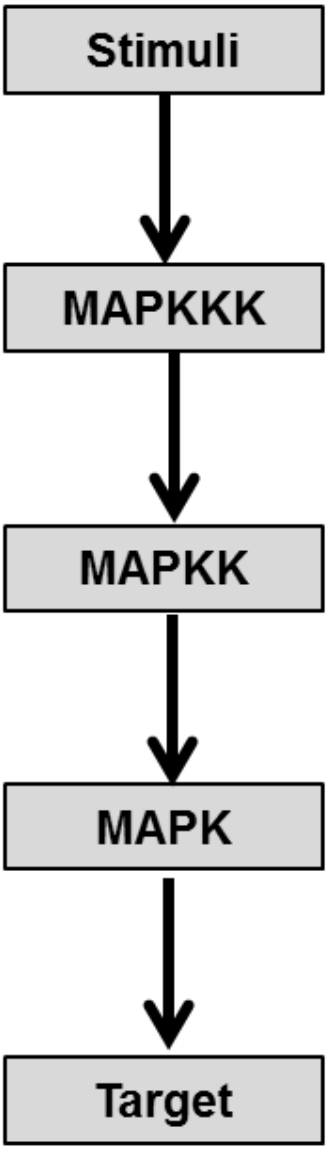

Figure 1.3: General structure of a mitogen-activated protein kinase signalling pathway
Each mitogen-activated protein kinase signalling pathway is composed of three kinases, mitogen-activated protein kinase kinase kinase (MAPKKK), mitogen-activated protein kinase kinase (MAPKK) and mitogen-activated protein kinase (MAPK) which act sequentially. Activation of the cascade, by various stimuli, results in the activation of targets in the nucleus or cytoplasm which ultimately brings about changes in cellular responses. (Adapted from Morrison, 2012).

1.3.2 Classification of MAPKs

Fourteen mammalian MAPKs have been characterised into seven different MAPK groups, based on sequence homology and substrate specificity (Cargnello & Roux, 2011; Dickinson & Keyse, 2006). MAPKs can be further classified into conventional or atypical MAPKs. c-Jun N-terminal kinases (JNK1/2/3), p38 kinases (p38α/β/γ/δ) and extracellular signal-regulated kinases (ERK1/2 and ERK5) are conventional MAPKs. Atypical MAPKs include: ERK3/4, ERK7/8 and nemo-like kinases (NLK). Classification into conventional and atypical MAPKs is based on the mechanism of activation by upstream signalling molecules (Coulombe & Meloche, 2007). Conventional MAPKs (JNK1/2/3, p38α/β/γ/δ, ERK1/2 and ERK5) contain the conserved Thr-X-Tyr motif in the activation loop of the kinase domain (Cargnello & Roux, 2011). For atypical MAPKs, ERK3/4 and NLK, a glycine or glutamic acid residue is present instead of a tyrosine residue (Coulombe & Meloche, 2007). The atypical kinase, ERK7, contains the conserved motif, Thr-Glu-Tyr in the activation loop, but there is a lack of evidence that ERK7 is phosphorylated by upstream molecules, rather ERK7 is activated by autophosphorylation (Abe et al., 2001). There is a greater understanding regarding the regulation, specificity and function of conventional MAPKs, compared to atypical MAPKs. Hence the conventional MAPKs, JNK1/2, p38 and ERK1/2 remain the focus in this thesis.

1.4 Extracellular signal-regulated kinase-1/2 (ERK1/2) signalling

1.4.1 Overview of ERK1/2 signalling pathway

The extracellular signal-regulated kinase-1/2 (ERK1/2) signalling pathway, also known as the MAPK/ERK pathway or Ras-Raf-MEK-ERK pathway, is the most characterised MAPK signalling pathway (Figure 1.4). ERK signalling plays a prominent role in cell survival and is involved in regulating differentiation, proliferation, growth, metabolism, cell migration and cell morphology (Shaul & Seger, 2007; Yoon & Seger, 2006).

The ERK signalling pathway is predominantly activated by a range of mitogenic stimuli including cytokines, phorbol esters and growth factors such as platelet-derived growth factor (PDGF), epidermal growth factor (EGF), nerve growth factor (NGF) and insulin-like growth factor 1 (IGF-1) (Cargnello & Roux, 2011). In addition, ERK signalling can be activated by cell stresses such as osmotic stress and microtubule depolymerisation (Raman, Chen, & Cobb, 2007). The mitogenic stimuli typically initiate ERK signalling by binding to, and activating cell surface receptors, for example growth factors typically activate receptor tyrosine kinases (RTKs) (Perona, 2006). Binding of the growth factor causes dimerisation of the receptor and subsequent autophosphorylation of the tyrosine residues in the cytoplasmic domain. The phosphorylated tyrosine residues bind a series of cytoplasmic adaptor proteins. Growth factor receptor-bound protein 2 (Grb2) binds to the phosphorylated tyrosine residues through a Src homology 2 SH2 domain and Grb2 also contains two SH3 domains which allows exchange factors such as son of sevenless (SOS) to bind to Grb2 (Margolis, Skolnik, Margolis, & Skolnik, 1994). This enables the recruitment of Ras proteins (H-Ras, K-Ras and N-Ras) to the membrane. Ras proteins are GTPases which are inactive when GDP-bound but conversion of GDP to GTP, catalysed by guanine exchange nucleotide factors (GEFs), results in active GTP-bound Ras (Omerovic, Laude, & Prior, 2007). Active Ras is able to recruit the MAPKKKs involved in ERK signalling to the membrane for activation.

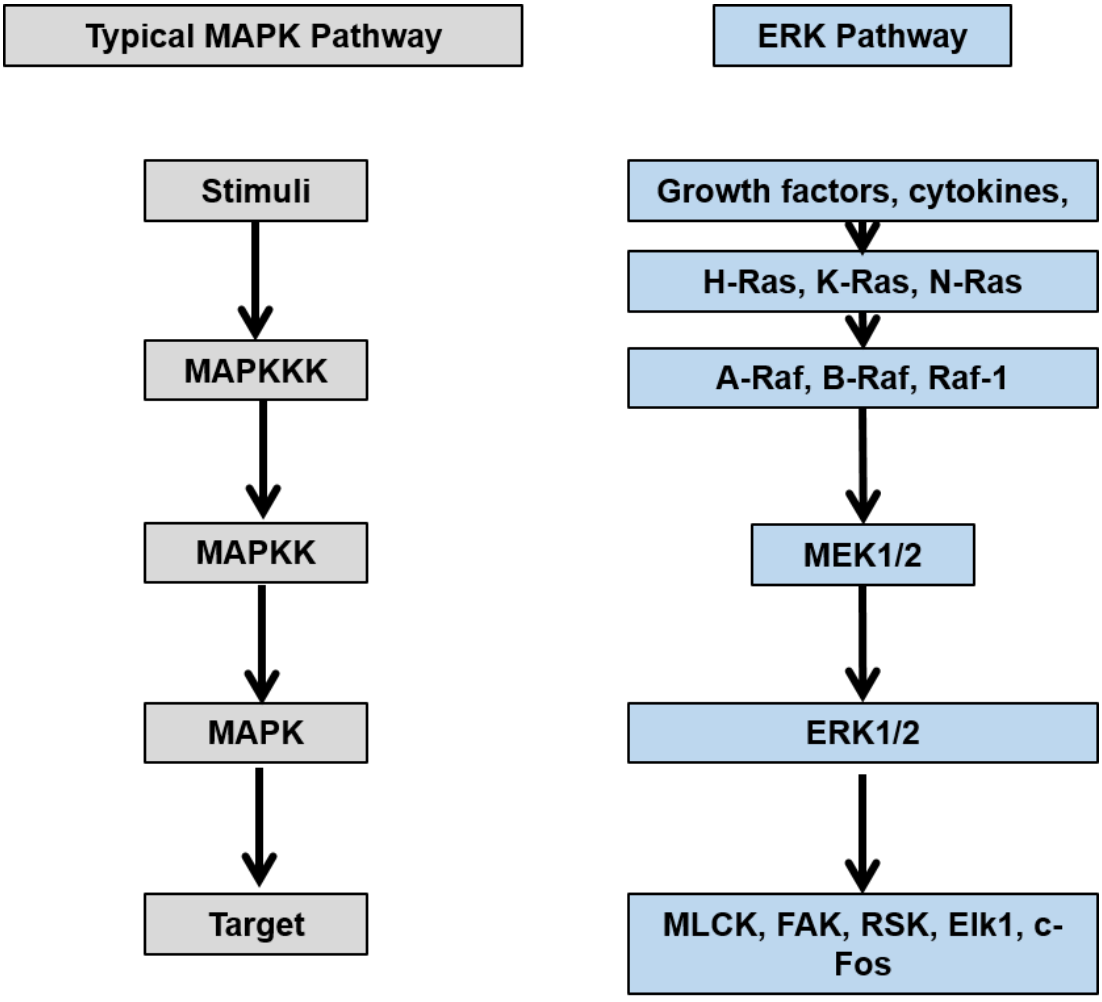

Figure 1.4: Generic structure of the ERK pathway
The ERK signalling pathway is activated by the binding of mitogenic stimuli to cell surface receptors Activation of cell surface receptors allows binding of adaptor proteins and exchange factors which facilitates the recruitment of Ras and subsequent Raf proteins. Activated Raf proteins phosphorylate and activate MEK1/2 which in turn phosphorylate ERK1/2. Activated ERK1/2 can activate over 200 cytoplasmic and nuclear targets.

The main MAPKKKs involved in ERK1/2 signalling are members of the Raf kinase family: A-Raf, B-Raf and C-Raf (Raf-1), with Raf-1 being the best characterised isoform (Wellbrock, Karasarides, & Marais, 2004). In addition to Raf kinases, other MAPKKKs involved in ERK signalling are Mos, tumour progression locus 2 (Tpl2) kinase and MAPK/ERK kinase kinase 1 (MEKK1) but the involvement of these MAPKKKs is restricted to certain cell types and stimuli (Raman et al., 2007; Wortzel & Seger, 2011). Mos functions in the reproductive system (Gotoh & Nishida, 1995), whereas Tpl2 functions mainly in transformed cells (Salmeron et al., 1996) and MEKK1 is involved in the response to stress stimuli (Lange-Carter, Pleiman, Gardener, Blumer, & Johnson, 1993).

The MAPKKs involved in ERK signalling are MEK1 (45 kDa) and MEK2 (46 kDa). MEK1 and MEK2 are structurally similar to other MEK proteins consisting of a catalytic kinase domain surrounded by a regulatory N-terminus region and a C-terminus region (Roskoski, 2012). MEK1 and MEK2 are 86% identical in sequence in the catalytic domain (Frémin & Meloche, 2010). Unlike other MEKs, MEK1 and MEK2 also contain a leucine-rich nuclear export sequence (NES) in the N-terminus region (Fukuda, Gotoh, Gotoh, & Nishida, 1996) and a proline-rich sequence in the kinase domain which is important for MEK regulation (Catling, Schaeffer, Reuter, Rukmini Reddy, & Weber, 1995). MEK1 and MEK2 are activated by the dual phosphorylation of serine residues within the conserved Ser-Xaa-Ala-Xaa-Ser/Thr motif within the activation loop of MEK1 and MEK2 (Raman et al., 2007; Shaul & Seger, 2007). Human MEK1 is phosphorylated at Ser218 and Ser222 whereas MEK2 is phosphorylated at Ser222 and Ser226 (Lavoie & Therrien, 2015).

ERK1 (44kDa) and ERK2 (42kDa), encoded for by the genes, *MAPK3* and *MAPK1* respectively, are the only known substrates of MEK1 and MEK2 (Shaul & Seger, 2007). ERK1 was the first mammalian MAPK to be characterised (Boulton et al., 1990) and later ERK1 and ERK2 were identified as kinases activated in response to insulin and nerve growth factor (NGF) (Boulton et al., 1991). MEK1 and MEK2 activate ERK1 and ERK2 through the dual phosphorylation of threonine and tyrosine residues within the Thr-X-Tyr motif in the activation loop of the ERK kinases (Payne et al., 1991; Shaul &

Seger, 2007). Human ERK1 is phosphorylated at Thr202 and Tyr204 whereas ERK2 is phosphorylated at Thr185 and Tyr187 (Lavoie & Therrien, 2015). ERK1 and ERK2 are ubiquitously expressed in all tissues but ERK2 is generally expressed at higher levels than ERK1 (Buscà, Pouysségur, & Lenormand, 2016). ERK1 and ERK2 are 83% identical in sequence (Cargnello & Roux, 2011).

Around 200 substrates of phosphorylated ERK1/2 have been identified, including both cytoplasmic and nuclear targets (Yoon & Seger, 2006). Cytoplasmic targets include proteins involved in regulating cell adhesion/migration (myosin light chain kinase-MLCK, paxillin and focal adhesion kinase-FAK), cell-cell communication (connexin 43-Cx43), cell death (death associated protein kinase-DAPK) and proteins involved in maintaining cytoskeletal structure (Ramos, 2008). Alternative cytoplasmic targets of ERK signalling are mitogen-activated protein kinase-activated protein kinases (MAPKAPKs). The main MAPKAPK activated by ERK signalling is ribosomal S6 kinase (RSK) (Sturgill, Ray, Erikson, & Maller, 2002). MAPK-interacting protein kinase (MNK) and mitogen- and stress-activated protein kinases (MSK) are additional MAPKAPKs activated by ERK1/2 (Deak, Clifton, Lucocq, & Alessi, 1998; Fukunaga & Hunter, 1997; Wortzel & Seger, 2011). Nuclear targets of phosphorylated ERK1/2 include transcription factors such as c-Jun, c-Fos, Elk-1 (Wortzel & Seger, 2011).

1.4.2 Role of ERK signalling in leukemogenesis

ERK signalling is involved in regulating the proliferation, differentiation and apoptosis of haematopoietic cells, allowing cells to respond to stimuli in the microenvironment of the bone marrow and blood. Early evidence showing the constitutive activation of ERK signalling in leukaemia cells highlighted the potential involvement of ERK signalling in leukemogenesis. Deregulation of ERK signalling allows immature or abnormal leukemic cells to proliferate, survive and avoid apoptosis.

Constitutive activation of ERK signalling has been reported in numerous studies, in both leukemic cell lines and primary leukemic cells. Out of 14 AML cell lines and 5 CML cell lines (from blast crisis) studied, significant

activation of MEK1/2 was observed in 8 and 2, of the AML and CML cell lines, respectively (Morgan, Dolp, & Reuter, 2001). ERK1/2 activation correlated with MEK1/2 activation; ERK1/2 activation was measured in 9 of the AML cells lines and 2 of the CML cell lines. Constitutive phosphorylation of ERK1/2 in the AML cell lines, NB4 and OCI-AML3, were confirmed in two alternative studies (Milella et al., 2001; Ricciardi et al., 2005). Constitutive activation of ERK1/2 has been reported in the majority (greater than 50%) of blasts from newly diagnosed AML patients (Kim et al., 1999; Milella et al., 2001; Ricciardi et al., 2005; Towatri et al., 1997). In addition to newly diagnosed AML patients, high levels of phosphorylated ERK1/2 have also been reported in samples from patients with relapsed or refractory AML (Ricciardi et al., 2005). Levels of phosphorylated ERK may have great prognostic value; high levels of phosphorylated ERK2 in AML cells is associated with a poor prognosis (Kornblau et al., 2006).

Constitutive activation of ERK signalling in leukemic cells is a result of either mutations in components of the ERK pathway, changes in the activity of phosphatases involved in regulating ERK signalling or changes in external stimuli, such as chemotherapy drugs. Mutations in MEK1/2 and ERK1/2 are rare and the constitutive activation of ERK signalling is primarily due to mutations in Ras proteins and to a lesser extent Raf proteins. Activating point mutations in Ras proteins, at codons 12, 13 and 61 are one of the most common genetic alterations in leukaemia, with varying frequencies among different leukaemia subtypes (Table 1.2). In leukaemia, mutations in N-RAS are more frequent than in K-RAS; H-RAS mutations are rare. Mutations in Ras proteins favour GTP-binding, producing a constitutively active GTP-bound form of Ras, promoting proliferative and survival signals (Prior, Lewis, & Mattos, 2012). In AML, mutations are common in the FMS-like tyrosine kinase 3 (FLT3) gene, which encodes for a receptor tyrosine kinase upstream of Ras (Table 1.2). FLT3 mutations can be categorised into two main types: internal tandem duplication (FLT3/ITD) mutations in the juxtamembrane domain of the receptor and point mutations in the activation loop of the tyrosine kinase domain (FLT3/TKD) (Levis, 2013; Levis & Small, 2003). Both mutations result in the constitutive activation of FLT3 and subsequent hyperactivation of Ras

and downstream kinases involved in ERK signalling which promotes leukemogenesis (Masson & Rönnstrand, 2009). Mutations directly in Ras proteins are extremely rare in CML. However in Philadelphia chromosome-positive CML, formation of the fusion BCR-ABL gene and consequent constitutive tyrosine kinase activity, results in increased levels of active GTP (Table 1.2) (Kurzrock, Jordan, Gutterman, & Talpaz, 1988; Zou & Calame, 1999).

Initially Raf proteins were only considered to have an important role in cancer development due to their effects downstream of Ras proteins. It later became apparent that Raf proteins can themselves be directly mutated, resulting in constitutively activate ERK signalling, independent of upstream Ras proteins. Although mutations in Raf proteins are very common in cancer, with B-Raf being mutated in around 8% of all cancers (Holderfield, Deuker, Mccormick, & Mcmahon, 2014), Raf mutations in leukaemia are rare and appear to be restricted to certain types of leukaemia. For example, the most common BRAF mutation, V600E, is found in almost all (>97%) cases of hairy cell leukaemia, a rare type of leukaemia (Ahmadzadeh et al., 2014; Blombery et al., 2012; Falini, Martelli, & Tiacci, 2016; Tiacci et al., 2011). In contrast, it is apparent that B-Raf is not commonly mutated in AML. B-Raf mutations were reported in only 4% of AML cases (Lee et al., 2004), and other studies examining a larger number of cases did not report any Raf mutations in AML cases (Smith et al., 2003; Trifa et al., 2012).

Although it has been well established that genetic mutations, such as those discussed in the Ras and Raf proteins contribute to leukemogenesis, there is an emerging role of phosphatases in the development of leukaemia. Altered phosphatase activity can deregulate ERK signalling, allowing constitutively active ERK signalling. Phosphatase of activated cells 1 (PAC1) was one of the first phosphatases to be implicated in leukemogenesis. The PAC1 gene is downregulated in acute leukemic cells compared to normal bone marrow cells and is associated with the constitutive activation of ERK in leukemic cells (Kim et al., 1999). The protein phosphatase 2A (PP2A) family of serine/threonine phosphatases involved in downregulating ERK signalling (Millwards, Zolnierowicz, & Hemmings, 1999), has also been identified as one

of the main families of phosphatases dysregulated in leukaemia. Inhibition of PP2A activity, and the consequent hyperactivation of ERK signalling and leukemic cell survival has been reported in various types of leukaemia (Arrizau, Pippa, & Oder, 2016; Cristóbal, Garcia-Orti, Cirauqui, Alonso, & Calasanz, 2011; Neviani et al., 2005). Inhibition of PP2A activity can be caused by either post-translational modifications to the enzymatic C subunit or through the activity of regulatory proteins which interact with the regulatory A and B subunits of PP2A (Ramaswamy, Spitzer, Kentsis, Ruvolo, & Gutierrez, 2015; Yang, Huang, Lu, Li, & Huang, 2012). Reactivation of PP2A activity in leukemic cells induces apoptosis (Gutierrez et al., 2014; Ramaswamy et al., 2015; Smith et al., 2016; Yang et al., 2012).

Table 1.2: Genetic alterations causing the constitutive activation of Ras in leukaemia
(Adapted from Reuter, Morgan, & Bergmann, 2000).

Leukaemia subtype	Mutation	Frequency (%)	References
Acute myeloid leukaemia (AML)	Point mutation of N-RAS, K-RAS	20-30	[1-4]
	FLT3/ITD or FLT3/TKD	20-30	[5-7]
Childhood AML	Point mutation of N-RAS, K-RAS	20-30	[3,8,9]
Acute lymphocytic leukaemia (ALL)	Point mutation of N-RAS, K-RAS	20	[10,11]
Childhood ALL	Point mutation of N-RAS, K-RAS	10-35	[8,12]
Chronic myeloid leukaemia (CML)	BCR-ABL t(9;22), Ras-GTP	90-95	[13-15]
Chronic lymphocytic leukaemia (CLL)	Point mutation of N-RAS, K-RAS	5-10	[16,17]

[1] Bos, 1989, [2] Bryne & Marshall, 1998, [3] Farr, Saikit, Erlicht, Mccormickf, & Marshall, 1988, [4] Janssen et al., 1987, [5] Gilliland & Griffin, 2002, [6] Levis, 2013, [7] Löwenberg et al., 2005, [8] Liang et al., 2006, [9] Vogelstein et al., 1990, [10] Browett & Norton, 1989, [11] Neri, Knowlest, Greco, Mccormickt, & Dalla-Favera, 1988, [12] Irving et al., 2014, [13] Bennour et al., 2012, [14] Onida et al., 2002, [15] Zou & Calame, 1999, [16] Doménech et al., 2012, [17] Kanagal-Shamanna et al., 2015.

1.4.3 Targeting ERK signalling in leukaemia

The constitutive activation of ERK signalling in leukemic cells, which occurs via a range of mechanisms, is essential for the survival of leukemic cells. The important role of ERK signalling in mediating leukemic cell survival has been highlighted by studies in which targeting ERK signalling, in particular inhibition of the pathway, has been shown to reduce the survival of leukemic cells. Inhibition of ERK signalling, by targeting MEK1 using PD98059 or PD184352, inhibited the growth of OCI-AML3, HL-60 and NB4 cells, AML cell lines with high levels of active ERK (Milella et al., 2001). An alternative MEK inhibitor, U0126, induced apoptosis in alternative AML cell lines (KG1a, THP-1, M07e, HEL and TF-1) (Kerr et al., 2003). Investigations of the effect of a range of ERK signalling inhibitors on the growth of a variety myeloid leukemic cell lines showed Ras inhibitors (FT-277 and FPT-3) and MEK inhibitors (U0126 and PD98059) resulted in growth inhibition of the majority of AML cell lines (HL-60, NB-4, THP-1, MUTZ-2/3 and OCI-AML2/5) and CML cell lines (K562, EM-2, JK-1, LAMA-84) tested (Morgan et al., 2001). The pro-apoptotic effects of ERK inhibitors were confirmed in NB-4 cells, the AML cell line which showed the highest sensitivity towards the inhibitors. It has also been shown that inhibition of MEK, using PD98059, inhibits the growth and induces apoptosis in primary AML cells with constitutively active ERK, but not in normal haematopoietic progenitor cells (Lunghi et al., 2003; Milella et al., 2001).

Collectively the studies discussed suggest the ERK signalling pathways are a promising therapeutic target for leukaemia. In particular, these studies have focused mainly on targeting ERK in AML cells. This is not surprising, as targeting components of the ERK signalling pathway downstream of Ras might be beneficial in AML therapy, where the exact mutations causing constitutive activation of ERK1/2, are not always known. This is in contrast to therapy for CML, where the majority of patients have the BCR-ABL fusion gene and constitutive tyrosine kinase activity, making tyrosine kinase inhibitors a more effective therapeutic approach for CML.

1.4.4 Role of ERK signalling in response to leukaemia therapy

Targeting ERK signalling alone is not the only potential therapy for AML. Targeting ERK signalling in combination with pro-apoptotic small molecules or conventional chemotherapeutic agents might provide a more effective treatment strategy. This approach has been taken in a limited number of studies. PD98059 sensitised AML cell lines (OCI-AML3, NB4 and HL60) to all-*trans* retinoic acid, a chemotherapeutic agent used in the treatment of AML. Treatment of HL-60 cells with PD98059 prior to cytarabine (Ara-C), a treatment for AML and ALL, increased the sensitivity of HL60 cells to Ara-C-induced apoptosis (Milella et al., 2001). ABT-737 is a Bcl-2 inhibitor which inhibits cell growth and induces apoptosis in AML cells; pre-treatment of OCI-AML3 cells with PD98059 increased the apoptotic effects of ABT-737 (Konopleva et al., 2006).

1.4.5 Inhibitors of ERK signalling

As there is promising evidence for the potential therapeutic targeting of ERK signalling, numerous ERK signalling inhibitors have, and continue to be developed. These include ERK inhibitors for use in *in vitro* studies to gain a greater understanding of ERK signalling in various processes and inhibitors which can be used therapeutically in humans. General strategies to target ERK signalling include: preventing Ras membrane localisation using farnesyltransferase inhibitors (FTIs), directly targeting the GTP-binding pocket of Ras, interfering with RAS-SOS or RAS-Raf interactions, inhibiting Raf, MEK or ERK (Samatar & Poulikakos, 2014). Targeting ERK signalling upstream of Raf has been a challenge and more extensive testing of such inhibitors is required. The focus has therefore been on the development of small molecule inhibitors to target Raf, MEK1/2 and ERK1/2 (Table 1.3)

Several Raf inhibitors, such as vemurafenib, dabrafenib and encorafenib have been developed (Table 1.3) Raf inhibitors appear to be particularly effective in patients with BRAF(V600E) and have therefore mainly been used to target ERK in melanoma skin cancer, where the frequency of BRAF(V600E) mutation rate is higher, and also in hairy cell leukaemia (Dietrich et al., 2016; Tiacci et al., 2015). In cells with wild-type BRAF, rather than inhibiting Raf, Raf

inhibitors cause the activation of Raf and downstream ERK signalling components (Halaban et al., 2010; Hatzivassilou et al., 2010; Poulikakos, Zhang, Bollag, Shokat, & Rosen, 2010). This makes Raf inhibitors unsuitable in types of leukaemia, such as AML in which the BRAF(V600E) is rare. MEK1/2 and ERK1/2 inhibitors are therefore more attractive targets for inhibition of ERK signalling in leukaemia.

The focus has been on the development of MEK1/2 inhibitors as MEK1/2 are attractive targets for ERK signalling inhibitors for two main reasons: MEK1/2 function downstream of Ras and Raf where the majority of mutations occur and ERK1/2 are the only known substrates of MEK1/2 and therefore MEK1/2 inhibitors are highly selective for ERK1/2 signalling. The first small molecule MEK1/2 inhibitor to be developed was PD98059, and then U0126 was later developed (Table 1.3). PD184352 was the first MEK inhibitor to enter clinical trials but was unsuccessful. PD184352 however, in addition to U0126 have been extensively used in *in vitro* studies, including studies on leukemic cells. More recently developed MEK inhibitors include: PD0325901, Trametinib, selumetinib, binimetinib and cobimetinib (Table 1.3), but have mainly been tested in melanoma cells.

Whilst the focus has been on the development of Raf and MEK inhibitors, there has been less progress on the development of ERK1/2 inhibitors. Initially it was believed that there would be no benefit of targeting ERK1/2 compared to MEK1/2 as ERK1/2 are the only substrates of MEK1/2 (Samatar & Poulikakos, 2014). However, emerging evidence suggests that Raf and MEK inhibitors can cause negative feedback and increased levels of active ERK, which indicates the potential for directly targeting ERK1/2. ERK inhibitors might also be beneficial in cells resistant to Raf or MEK inhibitors. Recently developed ERK inhibitors include: SCH772984, FR180204, VTX11e and GDC-0994 (Table 1.3). ERK inhibitors are still in the relatively early stages of development but could provide a more effective method of targeting ERK signalling in leukaemia in the future.

In this thesis, U0126 and PD184352 will be used to target ERK signalling as these inhibitors have been widely used and shown to be effective at inhibiting ERK signalling in leukemic cells.

Table 1.3: Inhibitors of ERK signalling

Inhibitor	Mechanism of action	Additional information	References
Raf inhibitors			
Vemurafenib (PLX4032)	Inhibitor of all RAF isoforms *in vitro*. Potent inhibitor of mutated B-Raf *in vivo*.	First B-Raf inhibitor to enter clinical trials. Approved for BRAF(V600E) melanoma	[1,2]
Dabrafenib (GSK2118436)	Potent ATP-competitive inhibitor of mutated B-Raf	Second BRAF inhibitor developed. Approved for BRAF(V600E) melanoma	[3,4]
Encorafenib (LGX818)	Potent inhibitor of mutated B-Raf	Currently in phase 3 trials in patients with BRAF(V600E) melanoma and colorectal cancer	[5.6]
MEK inhibitors			
PD98059	Non-ATP-competitive inhibitor which prevents the activation of MEK but does not inhibit MEK activity	Not suitable for clinical trials due to weak inhibitory effect *in vitro*, poor solubility and inadequate bioavailability	[7,8]
U0126	Inhibits MEK1/2 activation by binding to the inactive form of MEK1/2 and therefore prevents the recruitment of Raf-1	Not suitable for clinical trials but widely used in *in vitro* studies	[9,10]
PD184352 (CI-1040)	Selective non-ATP-competitive inhibitor of MEK1/2	Greater potency than PD98059 and U0126. First MEK inhibitor to enter clinical trials and demonstrate *in vivo* activity but trials terminated due to poor solubility and rapid clearance. Widely used in *in vitro* studies	[11-13]
PD0325901	Highly potent and specific non-ATP-competitive inhibitor of MEK1/2	A structural analogue of PD184352 with greater potency, improved solubility and increased bioavailability. Development stopped at phase 1 study due to retinal vein occlusion toxicities	[14,15]
Selumetinib (AZD6244, ARRY-142886)	Potent, highly selective non-ATP-competitive inhibitor of MEK1/2. Locks MEK1/2 into an inactive conformation allowing ATP to bind but prevents interactions required for catalysis and access to ERK activation loop	Well tolerated in phase I trial. Antitumor activity shown in phase II trial of patients with advanced melanoma	[16-19]
Trametinib (GSK1120212)	Reversible, highly selective allosteric inhibitor of MEK1/2 activation and kinase activity	Approved for BRAF(V600E) melanoma	[20-22]

Table 1.3: Inhibitors of ERK signalling
Continued

Inhibitor	Mechanism of action	Additional information	References
Binimetinib (MEK162)	Potent ATP non-competitive allosteric inhibitor of MEK1/2	Tested in phase 2 study and showed a response in melanoma cells harbouring NRAS or BRAF(V600E)	[23]
Cobimetinib (GDC-0973)	Binds to and inhibits the catalytic activity of MEK1	Used in combination with Vemurafenib in the treatment of melanoma	[24,25]
ERK inhibitors			
SCH772984	ATP-competitive inhibitor of ERK1/2. Inhibits ERK enzymatic activity and subsequent phosphorylation of S6 kinase. Also stabilises a conformational state of ERK which prevents activation by MEK1/2	Effective in RAS and B-Raf mutant cancer cells	[26,27]
FR180204	Potent, cell permeable ATP-competitive inhibitor of ERK1/2	Not yet extensively profiled and relatively limited usage	[28]
VTX11e	ATP-competitive inhibitor of ERK2. Binds to ERK2 and prevents phosphorylation of downstream substrates	Orally bioavailable.	[29]
GDC-0994	Highly selective inhibitor of ERK1/2	In early phase 1 clinical development	[30]

[1] Flaherty et al., 2010, [2] Yang et al., 2010, [3] Falchook et al., 2012, [4] Hauschild et al., 2012, [5] Li et al., 2016, [6] Stuart et al., 2012**,** [7] Alessi, Cuenda, Cohen, Dudley, & Saltiel‡, 1995, [8] Dudley, Pang, Decker, Bridgest, & Saltiel, 1995, [9] Duncia et al., 1998, [10] Favata et al., 1998, [11] Allen et al., 2003, [12] LoRusso et al., 2005, [13] Sebolt-Leopold et al., 1999, [14] Brown, Carlson, Loi, & Graziano, 2007, [15] LoRusso et al., 2010, [16] Davies et al., 2007, [17] Kirkwood et al., 2012, [18] Leijen et al., 2011, [19] Yeh et al., 2007, [20] Abe et al., 2011, [21] Gilmartin et al., 2011, [22] Leonowens et al., 2014, [23] Ascierto et al., 2013, [24] Choo et al., 2012, [25] Rice et al., 2012, [26] Chaikuad et al., 2014, [27] Morris et al., 2013, [28] Ohori et al., 2005, [29] Aronov et al., 2009, [30] Robarge et al., 2014.

1.5 c-Jun N-terminal kinase (JNK) signalling

1.5.1 Overview of JNK signalling pathway

JNK signalling, an alternative MAPK signalling pathway, is involved in a variety of cellular processes including, inflammation, cell proliferation, differentiation, metabolism and cell death (Figure 1.5) (Bubici & Papa, 2014). In contrast to ERK signalling, which is predominantly involved in regulating cell survival, JNK signalling plays a more prominent role in the induction of apoptosis in response to stress.

JNK signalling is mainly activated by stress stimuli such as environmental cell stressors (UV, hypoxia, heat shock and osmotic shock), pathogens (lipopolysaccharide, peptidoglycan) and chemotherapy drugs (Figure 1.5) (Bogoyevitch & Kobe, 2006; Derijard et al., 1994; Matsuda, Kawasaki, Moriguchi, Gotoh, & Nishida, 1995; Seki, Brenner, & Karin, 2012). Other stimuli which activate JNK signalling are cytokines (tumour necrosis factor, interleukin-1) and growth factors (transforming growth factor β, epidermal growth factor, platelet-derived growth factor) (Bubici & Papa, 2014; Seki et al., 2012; Westwick, Weitzel, Minden, Karin, & Brenner, 1994).

In response to stimuli, MAPKKKs involved in JNK signalling are activated by GTPases such as Ras protein family members (Raf MKKKs) or Rho family of GTPases (Cdc42, RhoA) (Chen & Cobb, 2006; Gallagher, Gutowski, Sternweis, & Cobb, 2004; Russell, Lange-Carter, & Johnson, 1995). Out of twenty MAPKKKs identified, fourteen are involved in the activation of JNK signalling (Johnson & Nakamura, 2007). MEKK1 was the first MAPKKK identified to have a role in JNK signalling (Widmann, Gerwins, Johnson, Jarpe, & Johnson, 1998). Other MAPKKKs involved in JNK signalling include: members of the mixed lineage kinase family (MLK1-3), MEK kinases (MEKK1-4), apoptosis signal-regulating kinases (ASK1/2), tumour progression locus 2 kinase (TPL2), dual leucine zipper kinase (DLK), serine/threonine-protein kinase TAO1/2 and transforming growth factor activated kinase 1 (TAK1) (Cargnello & Roux, 2011; Fanger, Gerwins, Widmann, Jarpe, & Johnson, 1997; Gallo & Johnson, 2002; Kyriakis & Avruch, 2001).

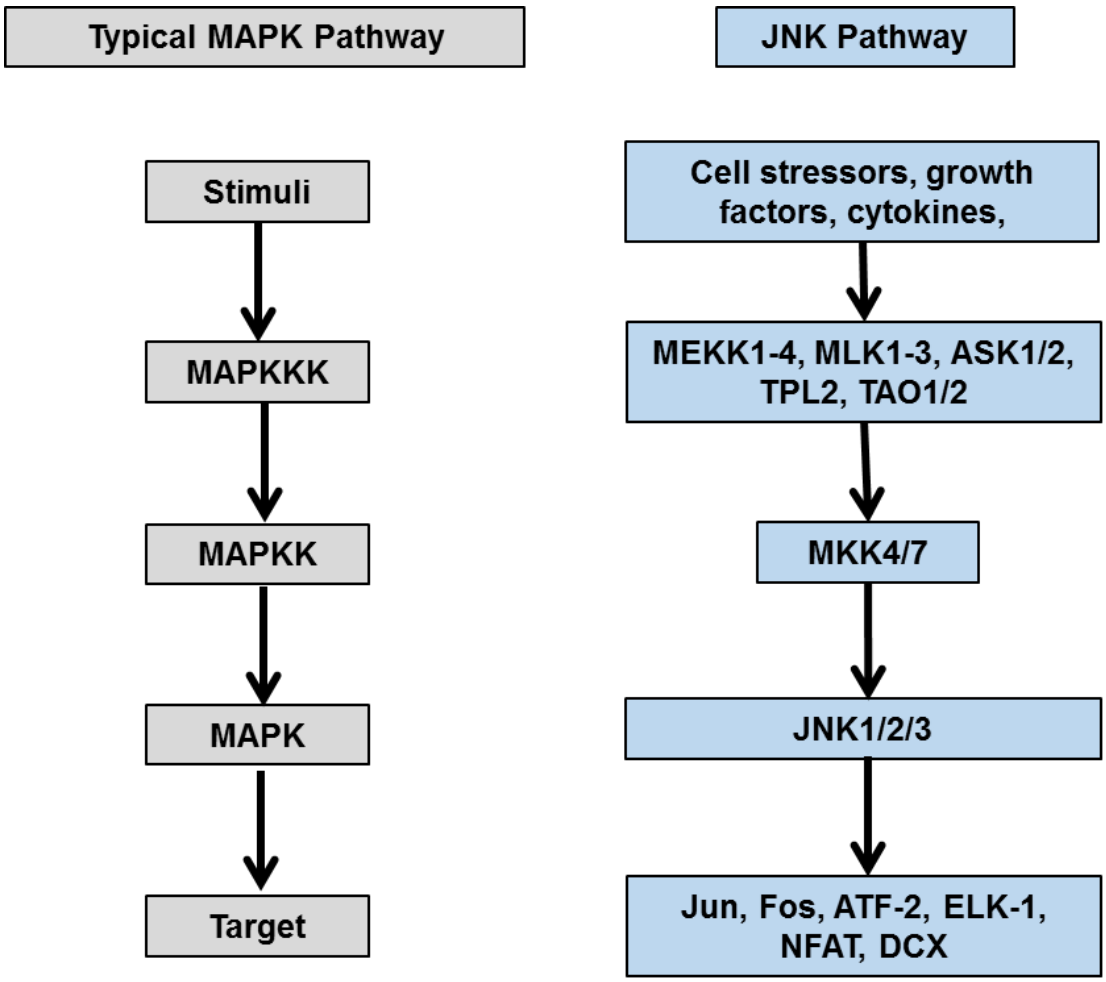

Figure 1.5: Generic structure of the JNK pathway
The JNK signalling pathway is activated by cell stressors, cytokines and growth factors which activate MEK kinases (MEKK), mixed lineage kinases (MLKs) and alternative MAPKKKs. Activated MAPKKKs phosphorylate and activate MKK4/7 which in turn phosphorylate JNK1/2/3. Activated JNK1/2/3 can activate over 50 nuclear and cytoplasmic targets.

In the JNK signalling cascade, MAPKKKs cause the phosphorylation of two main MAPKKs: MKK4 and MKK7 (Cargnello & Roux, 2011). MKK4 and MKK7 dually phosphorylate downstream JNK proteins at threonine 183 (Thr183) and tyrosine 185 (Tyr185) residues in the conserved Thr-Pro-Tyr residues in the activation loop of JNK. MKK4 shows preference for the phosphorylation of tyrosine whereas MKK7 shows preference for the phosphorylation of threonine and therefore MKK4 and MKK7 appear to function synergistically in the activation of JNK proteins (Lawler, Fleming, Goedert, & Cohen, 1998; Zou et al., 2007). Whilst MKK4 is specific for JNK signalling, MKK7 is involved in both JNK and p38 signalling (Haeusgen, Herdegen, & Waetzig, 2011).

In humans, there are three main JNK proteins, JNK1, JNK2 and JNK3, which are encoded for by *MAPK8*, *MAPK9* and *MAPK10*, respectively (Bubici & Papa, 2014). The JNK proteins are greater than 85% identical in sequence to each other (Cargnello & Roux, 2011). The diversity of JNK proteins arises from alternative splicing, which gives rise to at least ten different JNK isoforms (JNK1α1, JNK1α2, JNK1β1, JNK1β2, JNK2α1, JNK2α2, JNK2β1, JNK2β2, JNK3α1, JNK3α2) with molecular weights between 46 and 55 kDa (Bubici & Papa, 2014). JNK1 and JNK2 are ubiquitously expressed in most types of tissues whereas JNK3 expression is restricted to neuronal tissues in the brain and to a lesser extent in cardiac tissue, smooth muscle tissues and testes (Bode & Dong, 2007; Bogoyevitch & Kobe, 2006). Due to the restricted expression of JNK3, it is believed JNK3 may have a different function to JNK1 and JNK2; JNK1 and JNK2 however appear to have overlapping functions (Bode & Dong, 2007).

At least 50 different substrates of phosphorylated JNK are known, including both nuclear and non-nuclear proteins (Seki et al., 2012). c-Jun and other members of the Jun family of transcription factors are the main nuclear targets of activated JNK (Bogoyevitch & Kobe, 2006; Derijard et al., 1994). A Jun protein (c-Jun, JunB, JunD) dimerises with a Fos protein (c-Fos, FosB, Fra-1, Fra-2) to form the activator protein-1 (AP-1) transcription factor (Bubici & Papa, 2014). Activating transcription factor-2 (ATF-2) and ETS domain-containing protein (Elk-1) are alternative transcription factors activated by JNK

signalling (Cavigelli, Dolfi, Claret, & Karin, 1995; Van Dam et al., 1995). Other nuclear targets include nuclear factor of activated T cells (NFAT), c-Myc and p53 and nuclear hormone receptors such as the glucocorticoid receptor and retinoic acid receptor (Alarcon-Vargas & Ronai, 2004; Bogoyevitch & Kobe, 2006, 2006; Buschmann et al., 2001; Gupta, Campbell, Derijard, & Davis, 1995; Ortega-Pérez et al., 2005; Rogatsky, Logan, Garabedian, & Yamamoto, 1998; Yang, Whitmarsh, Davis, & Sharrocks, 1998). Non-nuclear targets include cytoplasmic cytoskeletal proteins (DCX, Tau) and apoptotic regulatory proteins (Bcl-2, Bad, Bax and Bim) (Bogoyevitch, 2006).

1.5.2 Role of JNK signalling in leukemogenesis

JNK signalling has different and opposing functions in cancer; there is evidence JNK can act as a tumour promoter and as a tumour suppressor (Bubici & Papa, 2014; Tournier, 2013). This is not surprising given the dual role of JNK signalling in regulating both cell survival and cell death. The JNK isoforms, JNK1 and JNK2 also have different and opposing functions in different types of cancer. The physiological and pathological function of JNK signalling depends on the stimuli, the cellular context, the JNK isoform involved, the strength and duration of JNK activation and hence the balance between JNK-mediated survival and JNK-mediated apoptosis (Gururajan et al., 2005; Platanias, 2003; Wagner & Nebreda, 2009). Whilst the role of JNK signalling in carcinogenesis appears to be well established in solid tumours, the role of JNK signalling in the development of haematological malignancies, such as leukaemia, is poorly understood.

There is limited evidence suggesting the role of JNK signalling in the development and progression of chronic leukaemias. High levels of activated JNK, in particular JNK1 have been reported in BCR/ABL-expressing CML cell lines (Burgess et al., 1998; Cripe et al., 2002). Constitutive BCR/ABL kinase activity results in activated JNK signalling in CML cells; inhibition of JNK signalling through the use of a dominant negative mutant of c-Jun, prevents the transforming ability of BCR/ABL (Raitano, Halpern, Hambuch, & Sawyers, 1995). Inhibition of JNK signalling, using a cytoplasmic JNK inhibitor, JIP-1, reduced cell proliferation and prevented the transforming activity of BCR/ABL

(Dickens et al., 1997). In particular constitutive BCR/ABL kinase activity results in the activation of JNK1 and inhibition of the JNK1 isoform prevents BCR/ABL-mediated transformation and survival of pre-B cells (Hess, Pihan, Sawyers, Favell, & Davis, 2002). JNK signalling is important for survival in BCR/ABL-expressing cells due to its ability to control Bcl-2 expression. The inability of BCR/ABL-expressing cells to survive in cells in which JNK signalling is inhibited, is due to decreased expression of Bcl-2; transgenic expression of Bcl-2 reverses the effects observed in JNK-inhibited cells. Collectively these studies suggest high levels of activated JNK play an important role in BCR/ABL-mediated transformation of haematopoietic cells.

There is also evidence that the constitutive activation of JNK signalling pathway plays an important role in the development of adult T-cell leukaemia (ATL) (Xu et al., 1996). Constitutive activation of JNK was observed in lymphocytes transformed *in vitro* by the human T cell leukaemia virus type 1 (HTLV-1) and in leucocytes from ATL patients. An alternative study has also reported the involvement of JNK signalling in HTLV-1-mediated ATL (Arnulf et al., 2002). Transforming growth factor-β1 (TGF- β1) signalling, a signalling pathway involved in the inhibition of T cell proliferation, is repressed by the Tax oncoprotein of HTLV-1 through a pathway involving the hyperactivation of JNK signalling; this implicates JNK signalling in ATL leukemogenesis.

In T-cell acute lymphocytic leukaemia (T-ALL) cell lines, low levels of activated JNK were measured, yet the low levels were required for survival of the T-ALL cells (Cui et al., 2009). Inhibition of JNK signalling in T-ALL cells, using the JNK inhibitors SP600125 and AS601245, induced cell cycle arrest and apoptosis in T-ALL cells. This study suggests that mechanisms, other than the hyperactivation of JNK may exist for the role of JNK signalling in leukemogenesis.

The studies discussed suggest JNK signalling is involved in the development and progression of different types of leukaemias. However the current understanding of the involvement of JNK signalling in leukaemogenesis appears to be restricted to these limited numbers of studies. Further investigations are needed to gain a more thorough understanding of the role of JNK signalling in leukemogenesis. Identifying the specific functions

of JNK signalling in certain types of leukaemia, will provide valuable information regarding how the JNK pathway can be targeted therapeutically to reduce survival and induce cell death in leukemic cells.

1.5.3 Role of JNK signalling in the response to leukaemia therapy

Activation of JNK signalling is a common event following chemotherapy treatment, and is often required for induction of apoptosis (Fan & Chambers, 2001). JNK signalling is activated in leukemic cells in response to a range of chemotherapeutic agents such as arsenic trioxide, paclitaxel and vincristine; the importance of JNK activation in mediating cell death induced by these chemotherapeutic agents however was not investigated (Bates, Lewis, Eastman, & Danilov, 2015; Davison, Mann, Waxman, & Miller, 2004; Peng et al., 2016; Shiah, Chuang, & Kuo, 2001). Activation of JNK signalling is essential for ceramide-induced apoptosis in K562 cells, a CML cell line (Nica et al., 2008). It has also been reported in K562 cells that PBOX-6, an anti-leukemic agent, causes the activation of JNK1 and JNK2 which is required for apoptosis induced by PBOX-1 (Mc Gee et al., 2002). Inhibition of JNK signalling prevents PBOX-1-induced apoptosis in K562 cells.

Failure to activate JNK signalling may therefore be a possible mechanism to explain the resistance of leukemic cells to chemotherapy. This has previously been shown to be the case in AML cells; failure to activate JNK signalling, conferred resistance to anthracycline chemotherapeutic agents (Lagadinou et al., 2008). The anthracyclines, daunorubicin and doxorubicin activate JNK signalling in U937 cells, in particular the activation of JNK1; JNK1 however is not activated in anthracycline-resistant derived U937 cells (U937R cells) in response to anthracycline treatment. Further work in primary blasts from AML patients demonstrated that for patients who showed chemoresistance to daunorubicin, JNK was not activated by daunorubicin in the primary AML blasts *in vitro*. These results are consistent with studies in solid cancers, which show failure to activate JNK signalling is associated with chemoresistance (Brozovic et al., 2004; Li et al., 2005, 2006).

In contrast, there is evidence high levels of activated JNK can contribute to chemoresistance in leukemic cells. Constitutive activation of JNK signalling

has been reported in HL60/ADR cells, an anthracycline resistant AML cell line (Cripe et al., 2002). Inhibition of JNK signalling, using JNP (a non-phosphorylatable form of c-Jun) reduced the resistance of the HL60/ADR cells to daunorubicin. In primary AML cells, there was a strong association between constitutive activation of JNK signalling and failure to respond to treatment. The constitutive activation of JNK also correlated with high levels of multi-drug resistance protein (MRP)-efflux in the primary blasts. JNK signalling inhibition reduced MRP-efflux in the HL60/ADR cells. JNK activation has also been implicated in mediating resistance of T-cell ALL cells to tetrandrine, an anti-leukemic drug (Liou et al., 2017). Constitutive activation of JNK signalling was reported in tetrandrine-resistant Jurkat cells compared to tetrandrine-sensitive Jurkat cells and there was no difference in the expression of P-glycoprotein (P-gp) between tetrandrine-resistant and tetrandrine-sensitive cells. Inhibition of JNK signalling reduced tetrandrine resistance in the tetrandrine-resistant Jurkat cells; this suggests JNK signalling mediates resistance of Jurkat cells to tetrandrine but not through P-gp expression. An alternative mechanism of JNK-mediated chemoresistance has been proposed in T-cell acute lymphocytic leukaemia (T-ALL) cells. JNK1/2 was constitutively activated in Sup-T1 cells, a T-cell ALL cell line resistant to etoposide (Leung et al., 2008). Activated JNK1/2 caused an increase in the degradation of Bim_{EL}, a proapoptotic protein (Leung et al., 2008). Inhibition of JNK signalling, using SP600125 increased levels of Bim_{EL} and increased etoposide-induced apoptosis. JNK-mediated degradation of Bim_{EL} is therefore a possible mechanism of chemoresistance in leukemic cells.

It appears that there is therefore conflicting evidence for the role of JNK signalling in the response of leukemic cells to chemotherapeutic agents. Whilst it has been demonstrated failure to activate JNK signalling is associated with chemoresistance (Lagadinou et al., 2008), others have argued that constitutive activation of JNK signalling contributes to chemoresistance (Cripe et al., 2002; Leung et al., 2008; Liou et al., 2017). However the mechanism by which constitutive activation of JNK might mediate chemoresistance is not fully understood. The opposing effects observed in different types of leukemic cells and in response to different chemotherapeutic agents, suggest the role

of JNK signalling in mediating chemoresistance in leukemic cells is cell- and agent-specific. Gaining a greater understanding of the role of JNK-mediated cell death in response to specific chemotherapeutic agents in specific cell lines will provide an insight into how the JNK pathway can be targeted therapeutically to increase the susceptibility of leukemic cells to chemotherapy-induced cell death.

1.5.4 Inhibitors of JNK signalling

Given the important role of JNK signalling in disease development, progression and response to chemotherapeutic agents, inhibitors have been developed to target JNK signalling. The focus was initially of the development of small molecule ATP-competitive inhibitors, but has more recently been on the design of peptide inhibitors and ATP-site directed covalent inhibitors (Table 1.4). (Cui, Zhang, Zhang, & Xu, 2007). JNK signalling inhibitors mainly target JNK kinases.

The majority of small molecule JNK inhibitors are ATP-competitive inhibitors which function by competing with ATP for the binding site of the kinase and thereby preventing the phosphorylation of downstream substrates (Bogoyevitch & Arthur, 2008; Siddiqui & Reddy, 2010). Around 40 different small molecule ATP-competitive JNK inhibitors have been identified (Cui et al., 2007). The most commonly used ATP-competitive JNK inhibitors are CEP1347, SP600125, AS601245, CC-401 and AS602801 (Table 1.4). These inhibitors have been successful in therapeutically targeting JNK signalling in diseases, in particular neurodegenerative diseases (Yarza, Vela, Solas, & Ramirez, 2015). SP600125 however is the most extensively used JNK inhibitor in *in vitro* studies, including those utilising leukemic cell lines such as U937 cells (Moon et al., 2008; Moon, Choi, & Kim, 2011; Park et al., 2008). The use of ATP-competitive inhibitors is limited by two main factors: lack of efficacy and poor selectivity. The efficacy of ATP-competitive inhibitors is reduced by high levels of intracellular ATP and therefore high concentrations of the inhibitor are needed in order to compete with ATP (Bogoyevitch, 2005; Bubici & Papa, 2014). In addition, ATP-competitive inhibitors display poor selectively as these inhibitors target the ATP-binding site which is highly conserved in protein kinases, making it difficult for the inhibitors to differentiate between the

different JNK isoforms and to distinguish between JNK kinases and other protein kinases (Bogoyevitch & Arthur, 2008; Bubici & Papa, 2014).

Targeting JNK, at a site other than the ATP-binding site of JNK, such as the substrate binding site or regulatory site of the kinase, provides a more specific mechanism of inhibition than ATP-competitive inhibitors (Bogoyevitch, 2005). Much attention has been given to the development of peptide inhibitors which act in substrate-competitive manner, binding directly to the substrate-binding site of JNK. The first examples of JNK peptide inhibitors were JIP-derived peptides. JNK activation is facilitated by a scaffolding protein, JNK-interacting protein-1 (JIP1), which simultaneously binds JNK and upstream signalling components (Willoughby, Perkins, Collins, & Whitmarsh, 2003). JIP-derived JNK peptide inhibitors include: TI-JIP, TAT-TI-JIP and D-JNKI-1 (Bogoyevitch, 2005). The first JIP-derived JNK peptide inhibitor designed was a truncated inhibitor based on JIP1 (TI-JIP), a short 10 residue peptide, the minimum residues of JIP required for binding to JNK (Barr, Kendrick, & Bogoyevitch, 2002; Bonny, Oberson, Negri, Sauser, & Schorderet, 2001). The non-permeable TI-JIP peptide was enhanced by attaching an N-terminal TAT protein transduction domain sequence from the human immunodeficiency virus to produce a permeable TAT-TI-JIP peptide (Bonny et al., 2001). Poor stability of this peptide led to further development and the production of a stable peptide, D-JNKI-1. More recently PYC71N, a peptide not derived from JIP, has been developed (Ngoei et al., 2011). PYC71N is a potentially useful inhibitor for investigating JNK-mediated substrate interactions. Peptide inhibitors are often reversible, but the design of novel, irreversible cysteine-directed covalent inhibitors, such as JNK-IN-8, has addressed this problem (Zhang et al., 2012). Low concentrations of peptide inhibitors are required to effectively inhibit JNK signalling, but the peptides are often subject to proteolytic degradation *in vivo*, which limits their use (Bogoyevitch, 2005).

The ATP-competitive inhibitor SP600125 and the novel covalent JNK inhibitor, JNK-IN-8 will be used to target JNK signalling in this thesis. Whilst SP600125 has extensively been used in previous *in vitro* studies, JNK-IN-8

has been used in a limited number of studies and there are no previous reports of its use in leukemic cells.

Table 1.4: Inhibitors of JNK signalling

Inhibitor	Mechanism of action	Additional Information	References
ATP-competitive JNK inhibitors			
CEP-1347 (KT7515)	ATP-competitive inhibitor of mixed lineage kinase family members including MLK3	JNK inhibitor widely used mainly in the treatment of neurodegenerative disorders	[1-4]
SP600125	Reversible ATP-competitive inhibitor of JNK1/2/3	Extensively used in *in vitro* studies and in animal models. Usage limited due to poor specificity towards JNK and clinical safety profile unknown	[5,6]
AS601245 (JNK inhibitor V)	Potent ATP competitive inhibitor of JNK1/2/3	Selective for JNK3 at low doses. Only inhibits c-Jun phosphorylation at high concentrations due to limited cell penetration. AS6001245 has neuroprotective properties	[7-9]
CC-401	Potent ATP-competitive inhibitor of JNK1/2/3	Second generation inhibitor based on the structure of SP600125 which shows greater selectivity than SP600125 for JNK. Phase 1 clinical trial of CC-401 in AML terminated. Mainly been used in renal injury models	[10,11]
AS602801 (Bentamapimod)	ATP-competitive inhibitor of JNK1/2/3	Orally bioavailable. Potential anti-cancer agent but requires further testing	[11,12]
Peptide JNK inhibitors			
TI-JIP (JIP-1)	Peptide inhibitor of JNK which competes for JIP1 binding site on JNK, inhibiting JIP1-JNK binding and preventing JNK substrate phosphorylation. Peptide binding also induces conformational changes which distorts the ATP-binding site of JNK, lowering the ATP binding affinity	Peptide based on residues 153-163 of JIP-1 scaffold protein. Poor cell permeability	[13-16]

Table 1.4: Inhibitors of JNK signalling
Continued

Inhibitor	Mechanism of action	Additional information	References
TAT-TI-JIP (JNK Inhibitor VII)	Cell permeable peptide inhibitor of JNK which competes for JIP1 binding site on JNK, inhibiting JIP-JNK binding and preventing JNK substrate phosphorylation	TI-JIP coupled to 10 residues derived from human immunodeficiency virus TAT protein sequence	[13,17]
D-JNKI-1 (XG-102)	Peptide inhibitor of JNK which competes for JIP1 binding site on JNK, inhibiting JIP-JNK binding and preventing JNK substrate phosphorylation	JIP-1 peptide synthesis in the reverse sequence from D-amino acids rather than L-amino acids. Improved intracellular stability of the peptide but decreased efficacy. Inhibition of JNK activity decreased 15-20-fold compared to JIP-1	[13,18]
TAT-c-Jun peptide	Cell permeable peptide inhibitor which competes directly with c-Jun for c-Jun-binding site on JNK, inhibiting JNK-cJun interaction	Peptide derived from the δ-domain of c-Jun	[19]
PYC71N	Cell permeable peptide inhibitor which inhibits the interaction of c-Jun with JNK1	Peptide interacts with inactive JNK1 and c-Jun but does not interact with active, phosphorylated JNK1	[20]
ATP-site directed covalent inhibitors			
JNK-IN-8 (JNK Inhibitor XVI)	Cell-permeable, irreversible inhibitor of JNK1/2/3. Forms a covalent bond with a conserved cysteine residue in JNK and prevents the phosphorylation of c-Jun	Used in a limited number of studies targeting JNK in head and neck squamous cell carcinoma and breast cancer	[21-24]

[1] Kaneko et al., 1997, [2] Maroney et al., 1998, [3] Maroney et al., 2001, [4] Saporito, Hudkins, & Maroney, 2002, [5] Bennett et al., 2001, [6] Bain, McLauchlan, Elliott, & Cohen, 2003, [7] Carboni et al., 2004, [8] Gaillard et al., 2005, [9] Carboni et al., 2008, [10] Ma et al., 2007, [11] Messoussi et al., 2014, [12] Okada et al., 2016, [13] Bonny, Oberson, Negri, Sauser, & Schorderet, 2001, [14] Barr, Kendrick, & Bogoyevitch, 2002, [15] Bogoyevitch, Boehm, Oakley, Ketterman, & Barr, 2004, [16] Heo et al., 2004, [17] Kato et al., 2008, [18] Bubici & Papa, 2014, [19] Holzberg et al., 2003, [20] Ngoei et al., 2011, [21] Zhang et al., 2012, [22] Ebelt & van Den Berg, 2013, [23] Li, Song, Ji, & Xu, 2013, [24] Xie et al., 2014.

1.6 p38 signalling pathway

1.6.1 Overview of p38 signalling

p38 signalling, an alternative stress-activated MAPK signalling pathway (Figure 1.6) was identified independently by three research groups (Freshney et al., 1994; Han, Lee, Bibbs, & Ulevitch, 1994; John Rouse et al., 1994). p38 signalling is involved in regulating a range of cellular processes, similar to those that are regulated by JNK signalling and include: cell differentiation, proliferation, inflammation and cell death (Ono & Han, 2000).

p38 signalling is therefore activated by similar stimuli as for JNK signalling: cytokines, cell stressors and growth factors (Figure 1.6). The cytokines involved in the activation of p38 signalling are typically proinflammatory cytokines such as tumour necrosis factor alpha (TNF-α) and interleukin-1 (IL-1) (Cargnello & Roux, 2011; Freshney et al., 1994; Pietersma et al., 1997). Environmental stressors which stimulate p38 signalling are UV light, heat shock, osmotic shock, ischaemia and DNA-damaging drugs (Bogoyevitch et al., 1996; Clerk, Fuller, Michael, & Sugden, 1998; Hazzalin et al., 1996; Moriguchi et al., 1996; Zou & Blank, 2017). p38 signalling is also activated, but to a lesser extent, by growth factors (Escós, Risco, Alsina-Beauchamp, & Cuenda, 2016).

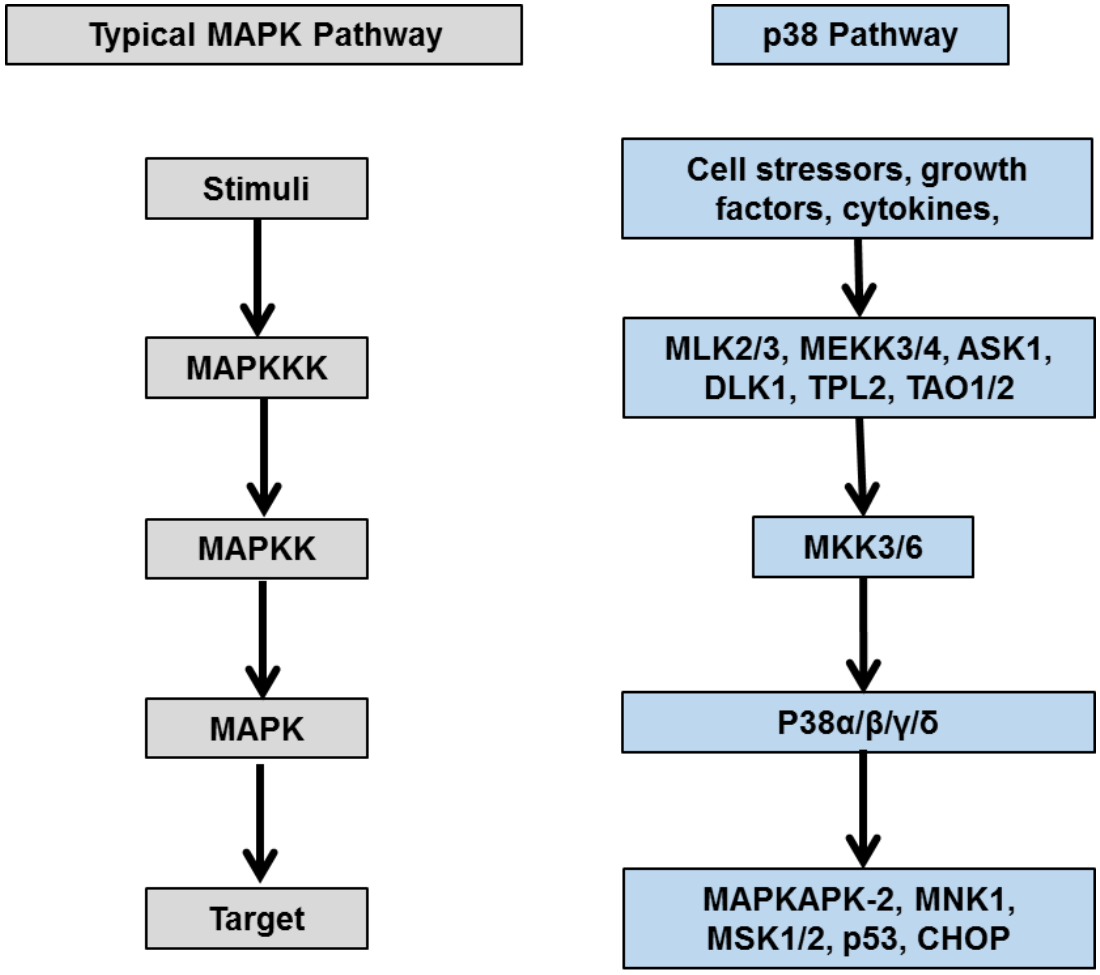

Figure 1.6: Generic structure of the p38 pathway
The p38 signalling pathway is activated by cell stressors, cytokines and growth factors which activate MEK kinases (MEKK), mixed lineage kinases (MLKs) and alternative MAPKKKs. Activated MAPKKKs phosphorylate and activate MKK3/6 which in turn phosphorylates p38α/β/γ/δ. Activated p38α/β/γ/δ can activate cytoplasmic targets and nuclear transcription factors.

In response to the stimuli, small GTP-binding proteins, such as Rac1 and Cdc42, are involved in the activation of the MAPKKKs involved in p38 signalling (Bagrodia, Dé, Davis, & Cerione, 1995; Zhang et al., 1995). MAPKKKS activated include: MLK2/3, MEKK3/4, ASK1, TAK1, DLK1, TPL2 and TAO1/2 (Cuadrado & Nebreda, 2010; Ichijo et al., 1997; Moriguchi et al., 1996; Zarubin & Han, 2005).

MKK3 and MKK6 are the MAPKKs responsible for the phosphorylation and activation of the p38 and are highly specific for p38 activation (Enslen, Raingeaud, & Davis, 1998). MKK4, a MAPKK mainly involved in the JNK signalling pathway, is also capable of activating p38α (Derijard et al., 1995). The p38 isoforms are differentially activated by MKK6 and MKK3; all four p38 isoforms are activated by MKK6 whereas MKK3 activates p38α, p38γ and p38σ but not p38β (Enslen et al., 1998). The activation of p38 MAPKKs is stimuli-specific. For example, MKK3 and MKK6 activates p38γ and p38β in response to environmental stressors (Remy et al., 2010). MKK6 is responsible for the activation of p38γ in response to tumour necrosis factor-α (TNF-α), whereas MKK3 activates p38σ in response to UV, hyperosmotic shock and anisomycin.

MKK3/6 are responsible for the activation of the p38 proteins by dually phosphorylating tyrosine and threonine residues within the conserved Thr-Gly-Tyr motif in the activation loop of the p38 protein (Cuadrado & Nebreda, 2010). In humans, there are four main p38 proteins: p38α, p38β, p38γ and p38σ which are encoded by the genes *MAPK14*, *MAPK11*, *MAPK12* and *MAPK13*, respectively (Zou & Blank, 2017). p38 proteins are divided into two subgroups, p38α/p38β and p38γ/p38σ; p38α and p38β are around 74% identical in sequence whereas p38γ and p38σ are 70% identical in sequence to each other (Cuenda & Rousseau, 2007; Escós et al., 2016; Jiang et al., 1996). The different p38 isoforms are widely expressed but display differential tissue expression. p38α is the best characterised protein and is ubiquitously expressed in the majority of cell types (Escós et al., 2016). p38β is predominantly expressed in brain tissue and also at high levels in thymus and spleen but is not expressed in skeletal muscle (Beardmore et al., 2005). p38γ expression is restricted to skeletal and heart muscle (Cuenda & Rousseau,

2007; Li, Jiang, Ulevitch, & Han, 1996). p38σ is expressed at high levels in endocrine glands such as those located in the pancreas, testes, kidney and small intestine (Escós et al., 2016; Kumar et al., 1997). The specific function of each p38 protein is not well established but it is known that each p38 has overlapping substrate specificity.

More than 100 downstream targets of p38 have been identified, which are mainly either kinases in the cytoplasm or transcription factors in the nucleus (Zou & Blank, 2017). MAP kinase-activated protein kinase 2 (MAPKAPK-2) was the first p38α substrate to be identified (John Rouse et al., 1994). Other protein kinases phosphorylated by p38 include: MK3, MAP Kinase Interaction Protein Kinase (MNK1), p38 regulated activated kinase (PRAK) and mitogen- and stress-activated kinase (MSK1/2) (Deak, Clifton, Lucocq, & Alessi, 1998; Fukunaga & Hunter, 1997; New et al., 1998; Ono & Han, 2000). Transcription factors activated by p38 include: activating transcription factor (ATF-1/2/6), tumour protein p53 and CCAAT-enhancer-binding homologous protein (CHOP) (Cuenda & Rousseau, 2007; Zou & Blank, 2017). Hsp27 and cycle proteins (cyclin D1, Cdc25) are alternative substrates of p38 (Cuadrado & Nebreda, 2010; Cuenda & Rousseau, 2007).

1.6.2 Role of p38 signalling in leukemogenesis

p38 signalling, plays an important role in the maintenance of haematopoiesis homeostasis, by tightly controlling the balance between proliferation and growth inhibition signals (Feng, Wen, & Chang, 2009; Porras & Guerrero, 2011). Deregulation of p38 signalling, can disrupt the balance and promote the development of haematopoietic malignancies, such as leukaemia. Similarly as for JNK signalling, the role of p38 signalling in leukemogenesis is less well understood than for ERK signalling.

p38 signalling is required for leukemic cell survival, in particular it is critical for the motility of leukemic cells which is essential for cell survival. Survival of leukemic cells is highly dependent on bone marrow stromal support (Manabe, Coustan-Smith, Behm, Raimondi, & Campana, 1992; Nishii et al., 1999; Panayiotidis, Jones, Ganeshaguru, Foroni, & Hoffbrand, 1996). The

interaction between the bone marrow stroma-secreted chemokine, stromal-derived factor-1α (SDF-1α/CXCL12) and the chemokine receptor type 4 (CXCR4) expressed on haematopoietic cells is important for leukemic cell migration (Bradstock & Gottlieb, 1995; Juarez et al., 2009; Perim et al., 2015). CXCR4 is highly expressed on progenitor ALL cells, making ALL cells more sensitive to CXCL12 than normal haematopoietic progenitors (Spiegel et al., 2004). p38 signalling is required for the CXCL12/CXCR4–mediated homing of B-cell ALL cells to the bone marrow; p38 signalling however is not involved in the CXCL12/CXCR4–mediated chemotaxis of normal haematopoietic progenitors (Juarez et al., 2009). Whilst p38 signalling is essential for chemotaxis of B-cell ALL cells, it was believed to not be required for the proliferation of primary B-cell ALL cells (Bendall, Baraz, Juarez, Shen, & Bradstock, 2005). However contradicting evidence suggests p38 signalling is in fact necessary for proliferation of ALL cells. Inhibition of p38 signalling, using the inhibitor SB203580, reduced the cytokine production of bone marrow stromal cells and reduced the stroma-mediated proliferation of ALL cells (Gaundar, Bradstock, & Bendall, 2009).

The constitutive activation of p38, as a result of the hyperactivation of MKK3/6 has been reported in B-CLL cells (Ringshausen et al., 2004). In agreement with the studies in B-cell ALL cells (Bendall et al., 2005; Juarez et al., 2009), p38 signalling is also important for the survival of B-CLL cells on bone marrow stromal cells (Ringshausen et al., 2004). In addition, p38 signalling is required for the production of metalloproteinase-9 (MMP-9) (Ringshausen et al., 2004). B-CLL cells produce metalloproteinase-9 (MMP-9) which is required for angiogenesis and homing to the bone marrow (Bauvois, Dumont, Mathiot, & Kolb, 2002). This suggests an alternative role of p38 signalling in leukaemogenesis: p38 is involved in leukaemia angiogenesis.

Collectively these studies suggest an important role of p38 signalling in the migration and survival of leukemic cells, in particular in lymphocytic leukaemias (ALL and CLL). Inhibition of p38 signalling may therefore be a useful therapeutic strategy to prevent the survival of ALL and CLL cells. The role of p38 signalling in myeloid leukaemias (AML and CML) however appears to be less well understood. p38 plays a critical role in tumour necrosis factor-

alpha (TNF-α)-mediated survival and proliferation of AML cells (Liu, Fan, Liu, Olashaw, & Zuckerman, 2000). Inhibition of p38 signalling, using the p38 inhibitor SB203580, inhibits the TNF-α supported growth of Mo7e cells (acute megakaryoblastic leukaemia cell line). It is evident that further research is needed in order to gain a greater understanding of the role of p38 in the survival of leukemic cells, in particular, in AML and CML cells.

1.6.3 Role of p38 in response to leukaemia therapy

Given the important role of p38 signalling in response to stress stimuli, such as chemotherapeutic agents, it is not surprising that similarly as for JNK signalling, p38 signalling has been implicated in the response of leukemic cells to therapy. Interferon-α (IFN-α), is a chemotherapeutic agent which inhibits the growth of BCR/ABL-expressing CML cells (Grumbach et al., 2001; Yanagisawa et al., 1998). p38 signalling plays an important role in the anti-leukemic effects of IFN-α in CML cells (Mayer et al., 2001). Treatment of KT-1 cells (a CML cell line) and primary progenitors from CML patients with IFN-α, results in the activation of p38. Inhibition of p38 signalling, using the p38 inhibitors SB202190 and SB203580, prevented the anti-proliferative effects of IFN-α in both the KT-1 cells and in the primary CML cells. Activation of p38 signalling is also essential in CML cells for the anti-leukemic effects of the tyrosine kinase inhibitors imatinib and dasatinib (Dumka et al., 2009; Parmar et al., 2004). Imatinib resulted in the activation of p38 in the BCR/ABL-expressing CML cell lines (KIT-1, EM2 and EM3 cells) and in primary granulocytes from CML patients (Parmar et al., 2004). Inhibition of p38 signalling in the primary CML cells, using SB203580 and SB202190, prevented the growth-inhibitory effects of imatinib. Dasatinib resulted in the activation of p38 in BCR/ABL-expressing CML cell lines (KBM7, K562 and BAF3/p210 cells) and also the activation of other p38 signalling components such as MLK3, MKK3/6 and MAPKAPK-2, as demonstrated in BAF3/p210 cells (Dumka et al., 2009). Inhibition of p38 signalling, using either the chemical p38 inhibitor SB203580 or knockdown of p38α using siRNA prevents dasatinib-induced apoptosis and cell cycle arrest and reverses the anti-proliferative effects induced by dasatinib. This highlights the important role of the p38α isoform. p38 is activated in KT-1 CML cells in response to arsenic

trioxide treatment (Giafis et al., 2006). Inhibition of p38 signalling, using SB203580, enhances arsenic-trioxide-induced apoptosis, by a pathway which appears to enhance the activation of JNK signalling. Taken together, these studies suggest p38 activation is essential for the anti-leukemic effects of several chemotherapeutic agents (IFN-α, imatinib, dasatinib and arsenic trioxide) in CML cells (Dumka et al., 2009; Giafis et al., 2006; Mayer et al., 2001; Parmar et al., 2004).

p38 signalling has also been implicated in the response of CLL cells to chemotherapeutic agents. p38 signalling is essential for apoptosis in B-cell chronic leukaemia (B-CLL) cells, induced by rituximab (anti-CD20 antibody) (Pedersen, Buhl, Klausen, Geisler, & Jurlander, 2002). Inhibition of p38 signalling using SB203580, prevents the activation of MAPKAPK-2, and reduces rituximab-induced apoptosis in primary B-CLL cells.

Activation of p38 is also important in AML cells for the anti-leukemic effects of chemotherapeutic agents; p38 is activated in NB-4 cells (acute promyelocytic leukaemia cell line) in response to all-*trans*-retinoic acid (Alsayed et al., 2001) and arsenic trioxide (Giafis et al., 2006; Verma et al., 2002). Inhibition of p38 signalling prevented arsenic trioxide-induced apoptosis in NB-4 cells (Giafis et al., 2006). p38 is also activated in response to cytarabine (Ara-C) and daunorubicin in AML cells and p38 activation is essential for cell death in chemosensitive patients induced by these chemotherapeutic agents (Maha, Cheong, Leong, & Seiw, 2009).

In addition to the role of p38 signalling in the response of leukemic cells to conventional chemotherapies, p38 has also been implicated in the response of AML cells to novel chemotherapeutic agents. TW-92, a novel anti-leukemic agent, activates p38 signalling and induces cell death in U937 cells (Hallak et al., 2009). The activation of p38 is essential for cell death induced by TW-92; inhibition of p38 prevented TW-92-induced apoptosis. In contrast, p38 signalling is involved in the resistance of primary AML cells to birinapant, a smac-mimetic, a group of agents currently in early trials as anti-leukemic agents (Lalaoui et al., 2016). p38 inhibition, overcomes the resistance of primary AML cells to birinapant; p38 inhibition increases TNF production induced by smac mimetics. This highlights the importance of understanding

the role of p38 signalling in response to specific chemotherapeutic agents in specific cells.

Although it is not always the case, it is evident that p38 activation is a common event following the treatment of leukaemic cells with chemotherapeutic agents, and p38 activation is essential for chemotherapy-induced leukemic cell death. Similarly as for JNK signalling, failure to activate p38 signalling, or constitutive activation of p38 are therefore possible mechanisms for chemoresistance. p38 signalling has been implicated in the chemoresistance of leukemic cells to cytarabine (Ara-C) (Lobo et al., 2005). In U937 cells, AML cells which have low basal levels of p38, the apoptotic effect of Ara-C requires the activation of p38. In contrast, in BCR/ABL expressing K562 cells, p38 is constitutively activated and p38 activation is not altered by Ara-C; Ara-C has minimal effect on the death of K562 cells. Development of novel therapies to enhance p38 activation in response to chemotherapeutic agents may be an approach to induce cell death in leukemic cells.

1.6.4 Inhibitors of p38 signalling

Although therapies to enhance p38 activation may be clinically beneficially, inhibitors of p38 signalling are important for *in vitro* studies, in order to gain an understanding of the role of the p38 pathway in leukemogenesis and in the response to leukaemia therapy. p38 signalling inhibitors have been developed which target the p38 pathway at the level of the p38 kinases. Given the important role of p38α in inflammation and cancer, the focus has been on designing p38 inhibitors which target the p38α. The majority of p38 inhibitors are small molecule ATP-competitive inhibitors (Table 1.5). Less attention has been paid to the development of peptide p38 inhibitors.

Small molecule p38 inhibitors can be broadly classified into two groups depending on the sites targeted on the p38 kinase: inhibitors which target only the ATP binding site and inhibitors which target the ATP binding site and/or an adjacent allosteric site (Akella, Moon, & Goldsmith, 2008). The first ATP-competitive molecules designed were small molecule pyridinyl imidazoles such as SB203580, SB202190 and SB2039063 (Table 1.5) Further ATP-competitive inhibitors have been more recently developed, such as VX-745,

papinod, skepinone-L and LY2228820, which are more selective than the initial pyridinyl imidazoles molecules. ATP-competitive p38 inhibitors however lack the desired efficacy and selectivity, as discussed for ATP-competitive JNK inhibitors (Section 1.5.4).

p38 inhibitors which target allosteric binding sites, provide a more selective method of inhibition. Adjacent to the ATP-binding site is an allosteric binding site, termed the DFG site as is contains the Asp-Phe-Gly motif at the N-terminus of the activation loop (Treiber & Shah, 2013). Dorapimod (BIRB-896) is a small molecule inhibitor which targets both the ATP-binding site and the DFG site. Binding of the inhibitor to the DFG site causes a conformational change in the activation loop of the p38 kinase, preventing ATP binding (Pargellis et al., 2002).

A limited number of peptide p38 inhibitors have been developed and the usage of these inhibitors appears to be restricted. Peptide p38 inhibitors also target different sites of the p38 kinase; peptide p38 inhibitors include TAT-MKK3b (targets docking groove), VWCS (targets ATP-binding site) and FWCS (targets allosteric binding site). Given the success of peptide JNK inhibitors, future research needs to focus on the development of novel peptide p38 inhibitors.

The small molecule p38 inhibitors, SB202190 and SB203580, which have been extensively used in previous *in vitro* studies in a wide range of cells, will be used to target p38 signalling in this thesis.

Table 1.5: Inhibitors of p38 signalling

Inhibitor	Mechanism of action	Additional information	References
ATP-site directed small molecule inhibitors			
SB203580	Inhibits p38 catalytic activity by competitively binding in the ATP pocket	Does not inhibit p38 activation. Inhibits phosphorylation of MAPKAPK-2 and HSP27. Most widely used p38 inhibitor in *in vitro* studies	[1-3]
SB202190	ATP-competitive inhibitor of p38α/β	Similar structure and specificity to SB203580. SB202190 does inhibit phosphorylation of p38 by upstream kinases	[2,3,4]
SB239063	Potent and selective ATP-competitive inhibitor of p38α/β	Second-generation inhibitor. 3-fold more selective than SB203580. No inhibition of p38γ/δ	[5,6]
VX-745	Potent ATP-competitive inhibitor of p38α/β	22-fold greater selectivity for p38α over p38β. No inhibition of p38γ	[7,8]
Pamapimod	ATP-competitive inhibitor of p38α/β enzymatic activity	No inhibition of p38γ/δ. 30-fold greater selectivity for p38α over p38β. No inhibition of p38γ/δ	[9,10]
Skepinone-L	Potent ATP-competitive inhibitor of p38α/β	First ATP-competitive inhibitor with excellent *in vivo* efficacy and selectivity. Minimal inhibition of p38γ/δ	[11,12]
LY2228820	Potent ATP-competitive inhibitor of p38α/β	Does not inhibit p38 activation. Inhibits the phosphorylation of MAPKAPK-2	[13]
Allosteric-site directed small molecule inhibitors			
Doramapinod (BIRB 796)	Interacts with allosteric binding site and ATP-binding site, causing a conformational change in the DFG site in the activation loop, which produces a structure unable to bind ATP	Potent inhibitor of p38α/β/γ/δ with slow dissociation rate. First p38 inhibitor to be tested in a phase 3 clinical trial	[14-16]

Table 1.5: Inhibitors of p38 signalling
Continued

Inhibitor	Mechanism of action	Additional information	References
Peptide inhibitors			
TAT-MKK3b	Cell permeable peptide inhibitor binds to p38α docking groove, inhibiting interactions between p38 and downstream substrates	Fusion peptide containing 12 residues from the docking sequence derived from MKK3b and 11 residues from HIV-TAT sequence. Prevents p38 phosphorylation by upstream kinases and prevents phosphorylation of downstream substrates	[17]
VWCS	Tetrapeptide inhibitor which targets the ATP binding site of p38α	Limited use in head and neck squamous cell carcinoma	[18]
FWCS	Peptide inhibitor which targets the allosteric-site of p38α	Limited use in head and neck squamous cell carcinoma	[19]

[1] Cuenda et al., 1995, [2] Young et al., 1997, [3] Kumar et al., 1997, [4] Nemoto, Xiang, Huang, & Lin, 1998, [5] Underwood et al., 2000, [6] Barone et al., 2001, [7] Duffy et al., 2011, [8] Haddad, 2001, [9] Hill et al., 2008, [10] Cohen et al., 2009, [11] Koeberle et al., 2012, [12] Storch, Gehringer, Baur, & Laufer, 2014, [13] Campbell et al., 2014, [14] Pargellis et al., 2002, [15] Regan et al., 2003, [16] Kuma et al., 2005, [17] Fu, Meng, He, & Gu, 2008, [18] Gill et al., 2013, [19] Gill et al., 2014.

1.7 Cell death

Cell death is defined as a process by which a cell loses the ability to function and is a critical process in the development and homeostasis of the haematopoietic system. Deregulation of cell death as a result of altered signalling pathways which control cell death, allow leukaemia cells to evade apoptosis and survive. The main types of cell death are: apoptosis (type 1 cell death), autophagy (type 2 cell death) and necrosis (type 3 cell death) (Shimizu, Yoshida, Tsujioka, & Arakawa, 2014). Apoptosis, and to a lesser extent necrosis, will be studied in this thesis.

1.7.1 Apoptosis

Apoptosis, a form of programmed cell death, is mediated by energy-dependent biochemical mechanisms which programme cells to die (Elmore, 2007). Morphological features of apoptosis include cell shrinkage and chromatin condensation (pyknosis) (Kerr, Wyllie, & Currie, 1972; Saraste & Pulkki, 2000). In the early stages of apoptosis, plasma membrane integrity is maintained but phosphatidylserine is exposed on the outer leaflet of the plasma membrane (Segawa & Nagata, 2015). In later stages, the nucleus fragments (karyorrhexis) and the plasma membrane protrudes (membrane blebbing) (Jian Hua, 2000; Saraste & Pulkki, 2000). The plasma membrane eventually buds off to form a separate membrane around nuclear fragments and cytoplasmic components, forming apoptotic bodies (Elmore, 2007). Apoptotic bodies are then engulfed by macrophages which degrade the contents. In the absence of phagocytosis, apoptotic cells and bodies are removed by secondary necrosis (late apoptosis/secondary necrosis), an autolytic necrotic process (Silva, 2010).

The two main apoptotic pathways are the extrinsic (death receptor-mediated) and the intrinsic (mitochondrial) pathways (Figure 1.7). The extrinsic and intrinsic pathways are activated by different stimuli but do no act in isolation; there is cross-talk between the pathways. The extrinsic apoptotic pathway is initiated by the binding of external ligands to death receptors (TNF-TNFR1, FasL-Fas, TRAIL-DR4) (Guicciardi & Gores, 2009). The intrinsic apoptotic pathway is activated by a range of stress stimuli including heat shock, UV-radiation, DNA damage and chemotherapeutic drugs, which

causes the release of factors from the mitochondria (Fulda & Debatin, 2006; Stephen & Green, 2010). Both the intrinsic and extrinsic apoptotic pathways are mediated by the action of caspases, enzymes which cleave proteins at aspartic acid residues (Riedl & Shi, 2004). Caspases are usually expressed as inactive procaspases, which once activated by cleavage, can activate other caspases, initiating a caspase cascade and irreversibly committing a cell to death. Caspases can be classified as either initiator caspases (caspases-2,-8,-9,-10) or executioner caspases (caspases-3, -6, -7) (Chen & Wang, 2002; Li & Yuan, 2008; Walsh et al., 2008).

1.7.2 Necrosis

In contrast to apoptosis, necrosis is an energy-independent form of cell death (Elmore, 2007). Necrosis typically occurs in response to exposure of cells to excessive external stressors, for example heat or infection (Festjens, Berghe, & Vandenabeele, 2006). Necrosis is characterised by morphological changes such as: organelle and cell swelling, formation of cytoplasmic vacuoles and loss of membrane integrity (Ziegler, 2004). Disruption of the membrane causes the release of intracellular contents, which can act as danger signals and activate an inflammatory response (Proskuryakov, Konoplyannikov, & Gabai, 2003).

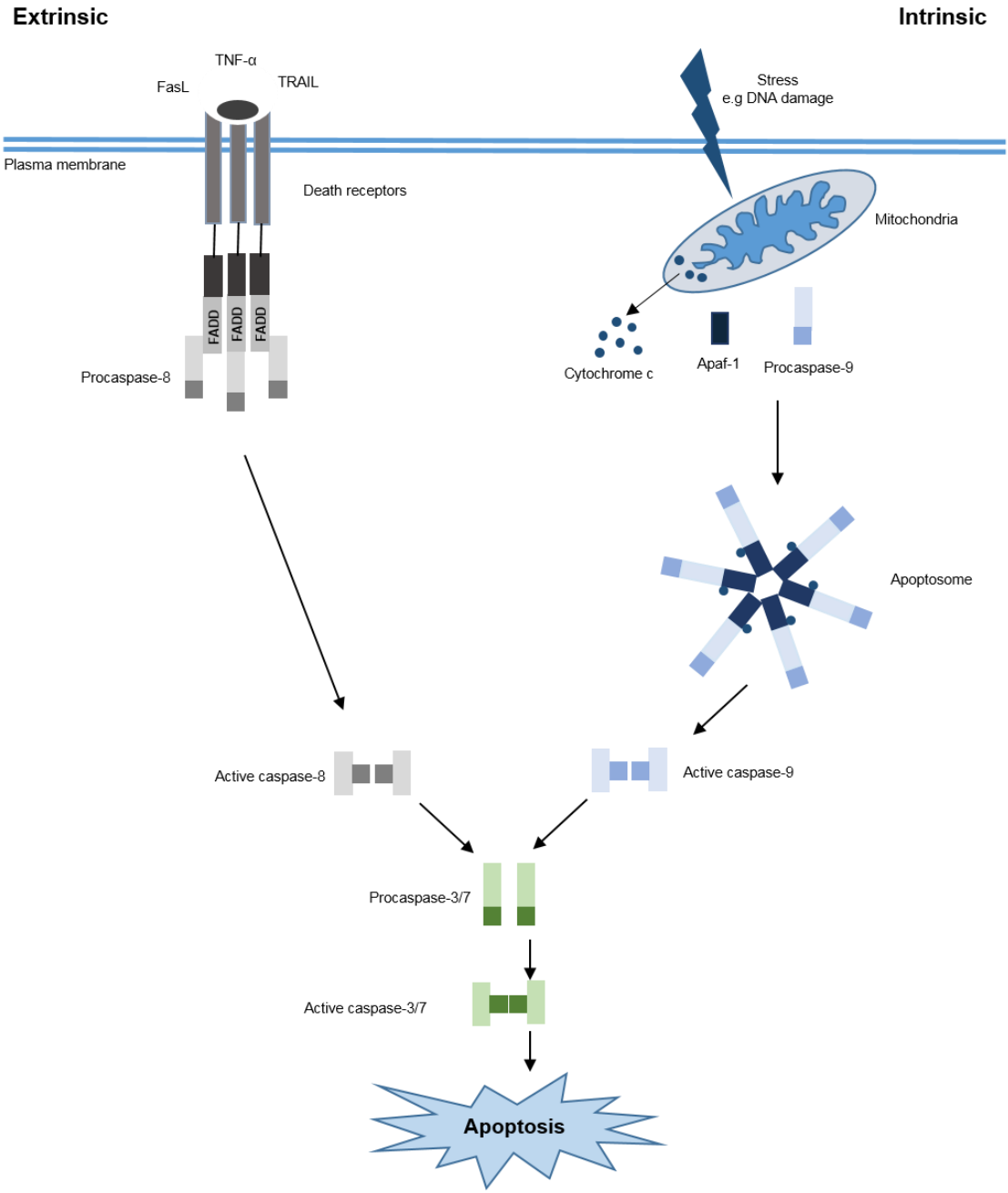

Figure 1.7: Extrinsic and intrinsic pathways of apoptosis
The extrinsic apoptotic pathway is initiated by the binding of ligands to death receptors. Ligand-receptor binding causes receptor oligomerisation and recruitment of procaspase-8 to the membrane via adaptor molecules such as Fas-associated death domain (FADD). The intrinsic apoptotic pathway is initiated by cell stresses which results in changes in mitochondrial membrane permeabilisation. This causes release of proapoptotic molecules, such as cytochrome c, from the mitochondrial intermembrane space into the cytosol. Released cytochrome c interacts with apoptotic protease activating factor 1 (Apaf-1), triggering assembly of the apoptosome and the subsequent activation of caspase-9. Active caspase-8 and caspase-9 can cleave and activate caspase-3 and caspase-7, leading to the induction of apoptosis. (Adapted from Cuda, Pope, & Perlman, 2016; Vuci, Dixit, & Wertz, 2011).

1.8 Experimental approaches for inducing cellular stress and cell death in leukemic cells

In order to investigate the role of MAPK signalling pathways in leukemic cell death, it is necessary to develop an experimental system for inducing cellular stress and/or cell death in leukemic cells. U937 cells, a human monocytic cell line for studying AML will be used in this thesis

Apoptosis can be induced by a range of stimuli: ultraviolet light, heat shock, hypoxia, nutrient deprivation, growth factors, cytokines, infection and chemotherapy drugs (Elmore, 2007). To gain a thorough understanding of the role of MAPK signalling pathways in leukemic cell death, the role of the pathways in response to a range of stimuli will be investigated. Ultraviolet light, heat and chemotherapeutic agents (doxorubicin and vincristine) are the cell stressors which will be investigated in this thesis and the reasons for selecting these cell stressors are outlined below.

1.8.1 Ultraviolet light

Radiation therapy is a type of cancer treatment which uses high-energy ionising radiation to kill cancer cells or shrink tumours. Radiation therapy induces cell death by causing DNA damage; the most lethal lesion generated by radiation is DNA double-strand breaks (DSBs) (Lomax, Folkes, & O'Neill, 2013; Santivasi & Xia, 2014). Chemotherapy is an alternative type of cancer treatment and many chemotherapeutic agents also function by causing DNA damage and inducing cell death (Kahlin Cheung-Ong, Giaever, & Nislow, 2013; Woods & Turhci, 2013). DNA-damage in cells initiates cell recovery mechanisms which are collectively termed the DNA-damage response (DDR) (Jackson & Bartek, 2009). The DDR involves DNA repair pathways (base excision repair, nucleotide excision repair, mismatch repair, double strand break repair), activation of cell cycle checkpoints and cell cycle arrest. If DNA damage cannot be repaired, cell death is induced. However, the DDR response is often defective in cancers, including leukaemia and cells with damaged DNA are able to progress through the cell cycle (Nilles & Fahrenkrog, 2017).

Ultraviolet (UV) light will be used as an apoptotic stimulus in this thesis as cell exposure to UV light is an ideal system to study cell death in response

to DNA damage. Treatment of cells with UV light generates reactive oxygen species (ROS) such as: singlet oxygen ($_1O^2$), superoxide radicals ($O_2•^-$), hydroxyl radicals (•OH), peroxyl radicals ($RO_2•$) and hydrogen peroxide (H_2O_2) (Prasad, Gupta, & Tyagi, 2017). The oxidative stress induced by UV light can result in DNA damage by causing single and double-stranded DNA breaks, tandem base lesions and by forming DNA-protein crosslinks (Avery, 2011; Cadet, Ravanat, Porro, Menoni, & Angelov, 2012). Oxidative stress also affects proteins and lipids by causing lipid peroxidation and protein oxidation (Avery, 2011).

1.8.2 Heat treatment

Heat treatment, in particular hyperthermia (temperatures are typically increased to between 40°C and 43°C) is an alternative cancer therapy which is effective at killing cancer cells and shrinking tumours. Sub-lethal heat treatments, which alone are often insufficient to cause cell death, are able to sensitise cancer cells to radiation therapy and to chemotherapy (Falk & Issels, 2001; Hildebrandt et al., 2002; Jolly & Morimoto, 2000). This means lower doses of radiation or chemotherapy are still effective and negative side effects are reduced. The therapeutic benefits of heat treatment have been established for a long time, but more recent research has focused on developing methods to deliver heat treatment specifically to cancer cells. Magnetic nanoparticles coated with targeting agents, which are heated using alternating magnetic fields have been successful in providing cancer cell-directed heat treatment (Chatterjee, Diagaradjane, & Krishnan, 2011; DeNardo & DeNardo, 2008; Giustini et al., 2010). Although utilisation of heat treatment has been established as an effective cancer therapy, the mechanisms associated with hyperthermia are not fully understood. It is known that exposure of cells to heat treatment results in cell cycle arrest at two main cell cycle checkpoints: G1/S and G2/M checkpoint (Kuhl & Rensing, 2000). Many chemotherapeutic agents, particularly microtubule-disrupting agents (Section 1.8.3) also function by causing cell cycle arrest and induction of apoptosis. Heat treatment will therefore be used as a cell stressor in U937 cells in this thesis. Heat treatment,

42°C for 1 h, has been selected for investigation based on previous work in the lab (Dempsey, 2009).

1.8.3 Chemotherapy

Chemotherapy is a type of cancer therapy which involves the use of one or more chemotherapeutic agents to induce death in cancer cells. The effectiveness of chemotherapy is limited due the ability of chemotherapeutic agents to non-specifically affect cancer and non-cancer cells; cancer cells can also evolve mechanisms to resist the chemotherapeutic agent, which is termed chemoresistance. As U937 cells are being utilised in this thesis, only chemotherapeutic agents used in the treatment of AML will be considered here.

Classes of chemotherapeutic agents used in the treatment of AML include: anti-metabolites, DNA-damaging agents, microtubule-targeting agents, monoclonal antibodies and FLT3 inhibitors (Table 1.6). The mainstay treatment for AML is cytarabine, an antimetabolite, in combination with an anthracycline. 50 to 75% of adults with AML achieve remission following combination treatment with cytarabine and an anthracycline (Tallman, Gilliland, & Rowe, 2005). Anthracyclines, such as daunorubicin, doxorubicin and idarubicin are DNA-damaging agents, which directly damage DNA (Cheung-Ong, Giaever, & Corey, 2013). Microtubule-targeting agents, such as the vinca alkaloids vincristine and vinblastine, inhibit microtubule polymerisation causing cells to undergo mitotic arrest (Moudi, Go, Yien, & Nazre, 2013). A greater understanding of the molecular changes involved in AML has led to the development of more targeted therapies, such as the monoclonal antibody gemtuzumab, which targets CD33, a molecule expressed on 80-90% of blasts in AML patients (Stadtmauer, 2002). Mutations in the FMS-like tyrosine kinase 3 (FLT3) occurs in a third of AML, and result in the constitutive activation of FLT3 which enables the survival of AML cells (Knapper et al., 2006). The FLT3 inhibitors, Sorafenib, Midostaurin, Lestauritnib and Quizartinib, are therefore novel chemotherapeutic agents for the treatment of AML (Larrosa-Garcia & Baer, 2017).

Doxorubicin and vincristine, two conventional chemotherapeutic agents used in the treatment of AML have been selected for investigation in this thesis. Doxorubicin and vincristine induce cell death via different mechanisms and therefore will provide a more thorough understanding of the role of MAPK signalling pathways in leukemic cell death induced by chemotherapy

Table 1.6: Chemotherapeutic agents used in the treatment of acute myeloid leukaemia (AML)

Drug	Mechanism of action	References
Anti-metabolites		
Cytarabine (cytosine arabinoside, Ara-C)	Inhibits DNA synthesis. Intracellularly converted to cytarabine triphosphate, which competes with deoxycytidine triphosphate for DNA polymerase	[1,2]
DNA-damaging agents		
Doxorubicin (**Adriamycin®**)	Intercalates into DNA and inhibits topoisomerase-II. Generation of free radicals and oxidative stress which causes cellular damage	[3,4]
Daunorubicin (Cerubidine)	Intercalation into DNA and inhibition of topoisomerase-II	[5,6]
Idarubicin (Idamycin)	Intercalation into DNA and inhibition of topoisomerase-II	[7,8]
Microtubule-targeting agents		
Vincristine	Binds to β-tubulin and inhibits microtubule polymerisation	[9, 10]
Vinblastine	Binds to β-tubulin and inhibits microtubule polymerisation	[10,11]
Monoclonal antibodies		
Gemtuzumab Ozogamicin (Mylotarg®)	Recombinant antibody which binds to CD33	[12,13]
FLT3 inhibitors		
Sorafenib	Inhibits FMS-like tyrosine kinase 3 (FLT3)	[14-16]
Midostaurin (PKC412)	Inhibits FLT3 kinase	[17,18]
Lestauritnib (CEP-701)	Inhibits FLT3 kinase	[19, 20]
Quizartinib (AC220)	Inhibits FLT3 kinase	[21,22]

[1] Löwenberg et al., 2011, [2] Momparler, 2013, [3] Schwartz, Preisler, & Kanter, 1981, [4] Yang, Teves, Kemp, & Henikoff, 2014, [5] Gong et al., 2015, [6] Luskin et al., 2016, [7] Carella, Berman, Marone, & Ganzina, 1990, [8] Bradstock et al., 2017 , [9] Ozgen, Savaan, Stout, Buck, & Ravindranath, 2000, [10] Moudi, Go, Yien, & Nazre, 2013, [11] Salerni, Bates, Albershardt, Lowrey, & Eastman, 2010, [12] Stadtmauer, 2002, [13] Giles & Estey, 2003, [14] Weiguo Zhang et al., 2008, [15] Pratz et al., 2010, [16] Fathi & Chen, 2011, [17] Stone et al., 2005, [18] Fischer et al., 2010, [19] Smith et al., 2004, [20] Knapper et al., 2006, [21] Cortes et al., 2013 [22] Levis, 2013.

1.9 Aims of the thesis

Deregulation of signalling pathways contributes to leukemogenesis. The MAPK signalling pathways, ERK, p38 and JNK are the focus of this thesis. It is evident constitutive activation of ERK signalling is involved in leukemogenesis by promoting cell survival and therefore inhibiting ERK signalling is an approach to induce cell death in leukemic cells. The role of ERK signalling is particularly well understood in AML cells, yet there is limited confirmation of the involvement of ERK signalling in the survival of U937 cells. In contrast to ERK signalling, the role of the JNK and p38 signalling pathways in leukemogenesis are less well understood and hence the p38 and JNK signalling pathways will be approached first in this thesis. There is limited evidence implicating JNK and p38 signalling in the progression and development of leukaemia, in particular AML. The JNK and p38 signalling pathways are activated by stress, such as chemotherapy so it is not surprising these pathways have been implicated in the response to leukaemia therapy. The evidence regarding the role of JNK and p38 in response to therapy however is contradictory. A number of studies suggest constitutive activation of JNK or p38 signalling mediates chemoresistance whereas others suggest failure to activate JNK or p38 signalling contributes chemoresistance. It is likely that the role of JNK and p38 signalling in leukemic death in cell-specific and agent-specific, but this requires further investigations.

Therefore, the overall aim of this thesis is to investigate the role of MAPK signalling pathways in leukemic cell death using the U937 model cell line.

The objectives of this thesis are:

- To optimise the treatment of U937 cells with cell stressors
- To determine the role of the JNK signalling pathway in U937 cell death
- To determine the role of the p38 signalling pathway in U937 cell death
- To determine the role of the ERK signalling pathway in U937 cell death
- To determine the role of the MAPK signalling pathways in *ex vivo* peripheral blood monocytes from healthy humans

Chapter 2

Materials and Methods

Chapter 2: Materials and Methods

2.1 Cell culturing

All cell culturing was performed in a class II tissue culture hood using aseptic technique to ensure sterility. Cells were maintained at 37°C in a humidified environment, supplemented with 5% CO_2.

2.1.1 U937 cell line

2.1.1.1 Subculturing of U937 cell line

Human caucasian histoyctic lymphoma, U937 cell line was purchased from European Collection of Cell Cultures (85011440). U937 cells were cultured in RPMI-1640 medium supplemented with 10% FBS. Prior to subculturing, cell viability was determined using the trypan blue exclusion test (Section 2.1.3). Cells were passaged every 3 to 4 days to maintain a cell density between 1 x 10^5 and 8 x 10^5 cells/mL.

2.1.1.2 Cryopreservation of U937 cell line

Cryopreservation of cell lines was carried out to enable stocks of cells to be stored. It avoids loss by contamination, reduces the cell passage number and subsequent genetic drift. Cells were frozen down in the log phase of growth (5 x 10^5 cells/mL) and no microbial contamination was present. Cells were centrifuged at 500 *g*, 25°C for 5 min. Following the removal of the supernatant, the cell pellet was gently re-suspended in 1 mL ice-cold freeze medium (90% FBS + 10% DMSO) in a cryogenic storage vial. Cryovials were incubated in liquid nitrogen vapour phase for at least 2 h before being transferred to liquid nitrogen in the cryostat at -196°C. Cells were cryopreserved until required.

2.1.2 Peripheral blood mononuclear cells (PBMCs)

2.1.2.1 Blood collection and processing

Ethical approval (Application 1204/16/MC/IoM) was obtained from the Faculty of Medicine, Dentistry & Life Sciences Research Ethics Committee. Participants were informed of the details of the study and each participant provided informed consent. Whole blood was collected from healthy volunteers aged between 18 and 60 years by venepuncture in 10 mL Vacutainer EDTA tubes. A full blood count on whole blood was performed using DxH 500 Haematology System.

2.1.2.2 Isolation of PBMCs from whole blood

PBMCs were isolated from whole blood by density centrifugation using Histopaque-1077. Freshly collected blood (3 mL) was added to an equal volume of DPBS without Ca^{++} or Mg^{++} (3 mL) and mixed by gently inverting the tubes several times. The blood-DPBS mixture (3 mL) was carefully overlaid on Histopaque-1077 (3 mL) which had been equilibrated to room temperature in 15 mL centrifuge tubes. The blood was centrifuged at 400 *g* (without brake*)*, room temperature, for 30 min. Using a Pasteur pipette, the upper layer, containing plasma, was removed to within 0.5 cm of the opaque interface. The opaque interface, containing PBMCs, was collected using a Pasteur pipette and transferred to a fresh 15 mL centrifuge tube containing DPBS (10 mL). The PBMCs were gently mixed and then centrifuged at 250 *g,* room temperature, for 10 min. The supernatant was removed and the PBMC pellet was re-suspended in 5 mL DPBS. PBMCs were centrifuged at 250 *g,* room temperature, for 10 min. The DPBS was discarded and the DPBS wash was repeated once more. After discarding the supernatant, the cell pellet was re-suspended in complete RPMI supplemented with 10% FBS and 1% antibiotic solution and a cell count was performed using trypan blue exclusion assay (Section 2.1.3). A full blood count was also performed post-isolation.

2.1.3 Cell counting and viability testing

Trypan blue exclusion assay was performed to determine cell concentration and cell viability. Typically, 50 µL of cell suspension was diluted 1:1 in 0.4% trypan blue solution. The sample was incubated with the dye at room temperature for around 2 min (incubations exceeding 10 min can cause decreased viability due to trypan toxicity). A small amount of cell-trypan blue solution was added to both chambers of a haemocytometer. Cells were visualised microscopically using 10 X objective; live cells possess intact cell membranes which exclude the dye whereas non-viable cells take up the dye and stain blue.

2.1.4 Preparation of cells for treatment

Around 24 h prior to treatment, U937 cells were subcultured, typically using a 1:1 split ratio, to ensure the cells were in the log phase of growth for treatment. Before use in experiments, the cell count was adjusted to 5 x 10^5 cells/mL by centrifuging the cells at 500 *g*, room temperature, for 5 min and re-suspending the cell pellet in the appropriate volume of complete RPMI.

2.2 Cell treatments

2.2.1 MAPK inhibitors

Sterile stock solutions of MAPK inhibitors were prepared as shown in Table 2.1. Under sterile conditions, dilutions of each MAPK inhibitor were prepared fresh before each experiment by diluting stock MAPK inhibitor in the appropriate volume of complete RPMI media.

Table 2.1: Stock solutions of MAPK inhibitors

Inhibitor	Manufacturer	Product number	Stock concentration (mM)	Solvent	Storage (°C)
SP600125	Sigma-Aldrich Co.	S5567	50	DMSO	4
JNK-IN-8	Selleck Chemicals	420150	10	DMSO	-20
SB202190	Sigma-Aldrich Co.	S7076	50	DMSO	4
SB203580	Sigma-Aldrich Co.	S8307	25	DMSO	-20
PD184352	Sigma-Aldrich Co.	PZ0181	50	DMSO	20
U0126	Sigma-Aldrich Co.	U120	25	DMSO	4

2.2.2 UV light treatment

The UVP Ultra Violet Product™ Variable Intensity Transilluminator was allowed to warm up for 15 min prior to use. Cells were exposed to UV light for 0-50 s and then incubated at 37°C, 5% CO_2 for 24 h.

2.2.3 Preparation of drug treatments

Stock solutions of drugs were prepared under sterile conditions as shown in Table 2.2. Dilutions of the drug treatments were prepared fresh before each experiment, by diluting the stock solution in the appropriate volume of complete RPMI-1640 media. Cells were typically treated with between 0 and 200 µM doxorubicin and between 0 and 200 nM vincristine.

Table 2.2: Stock solution of drug treatments

Drug	Manufacturer	Product number	Stock concentration (mM)	Solvent	Storage (˚C)
Doxorubicin	Sigma-Aldrich Co.	D1515	10	dH_2O	- 20
Vincristine	Sigma-Aldrich Co.	V8879	5	DMSO	- 20

2.2.4 Heat treatment

Heat treatment was performed by incubating cells at 42°C in a circulating water bath for 1 h. Control cells were incubated at 37°C, 5% CO_2 in an incubator for 1 h.

2.2.5 Anisomycin treatment

As anisomycin is an inducer of apoptosis and an agonist of MAPK signalling, it was used in experiments to act as a positive control for the induction of MAPK signalling. A stock of anisomycin was prepared under sterile conditions as shown in Table 2.3. Dilutions of anisomycin were prepared fresh by diluting the stock solution in the appropriate volume of complete RPMI-1640 media. Cells were typically treated with 1 µM anisomycin for 30 min.

Table 2.3: Stock solution of anisomycin

Drug	Manufacturer	Product number	Stock concentration (mM)	Solvent	Storage (˚C)
Anisomycin	Sigma-Aldrich Co.	A9789	25	DMSO	4

2.3 Methods to assess MAPK phosphorylation

2.3.1 Polyacrylamide gel electrophoresis and Western blotting

2.3.1.1 Cell lysis and protein extraction

This protocol was carried out to extract proteins from the whole cell. Cells were transferred to centrifuge tubes and centrifuged at 500 *g*, room temperature, for 5 min. Following the removal of the supernatant, cells were re-suspended in 1 ml ice-cold PBS and centrifuged again at 500 *g*, 4°C for 5 min. Samples were kept on ice at all times to avoid dephosphorylation. The supernatant was removed and the cell pellet was vigorously suspended in ice-cold cell extraction buffer (150 mM NaCl, 1% Triton X-100, 50 mM Tris, pH supplemented with Halt™ Protease and Phosphatase Inhibitor Cocktail). Cells were lysed on ice for 30 min with occasional agitation and then centrifuged at 13,500 *g*, 4°C for 20 min. The supernatant was collected and transferred to a fresh centrifuge tube. Where possible, the sample was aliquoted to avoid freeze-thaw cycles and stored at -80°C. The quantity of protein extracted was determined using bicinchoninic acid protein assay (Section 2.3.1.2).

2.3.1.2 Determination of protein concentration

Many detergents interfere with traditional Bradford assay reagents such as Coomassie, by inhibiting colour development and causing precipitation. Due to the high TritonX-100 content in the cell extraction buffer, a Bradford assay could not be used to determine the protein concentration in samples. Bicinchoninic acid (BCA) protein assay, an alternative detergent-compatible protein assay was used.

The BCA protein assay was performed using Pierce™ BCA Protein Assay Kit, following the kit instructions for microplates. BCA protein

standards (0-2000 µg/mL) were prepared fresh in cell extraction before each assay. The BCA working reagent was prepared freshly by mixing 50 parts of reagent A with 1 part of reagent B. 2.5 µL of standard or unknown sample and 20 µL of working reagent were added in triplicate to each well of a 384-well microplate. The plate was shaken on a plate shaker for 30 s and then incubated at 37°C for 30 min. After allowing the plate to cool to room temperature, the absorbance was measured at 562 nm using Varioskan™ LUX multimode microplate reader.

The protein concentrations of the unknown samples were determined using the standard curve. If the protein concentration of the sample was not within the working range, samples were diluted in extraction buffer until within range.

2.3.1.3 Sample preparation

The concentrations of the samples were adjusted in extraction buffer to ensure all samples contained equal concentrations of total protein. The samples were then diluted 1:1 in 2X reducing sample buffer (62.5 mM Tris-HCl, pH 6.8, 20% glycerol, 2% SDS, 0.01% bromophenol blue, 150 mM DL-dithiotreitol). The samples were heated to 85°C for 10 min. Samples were cooled for at least 10 min before loading onto the gels.

2.3.1.4 SDS-PAGE electrophoresis

Proteins were separated by sodium dodecyl polycacrylamide gel electrophoresis (SDS- PAGE), using an acrylamide concentration appropriate for the size of the target protein. 4-15% Mini-PROTEAN®TGXTM Precast Protein Gels (Bio-Rad Laboratories, Inc.) were used or discontinuous polyacrylamide gels were cast using Fisherbrand™ Vertical Gel Electrophoresis System, according to manufacturer's instructions. The resolving gel solution was prepared by combining all reagents in Table 2.4 (except APS and TEMED), degassing under vacuum for 15 min and then adding APS and TEMED. Once set, the stacking gel was prepared in a similar way (Table 2.5).

Table 2.4: Recipe for 12. 5% resolving gel
* enough for two 1 mm spaced gels

Reagent	Volume
1.5 M Tris HCl pH 8.8	3.75 mL
dH_2O	4.8 mL
Acrylamide	6.25 mL
10% SDS	150 µL
10% APS	50 µL
TEMED	50 µL

Table 2.5: Recipe for 3% stacking gel
* enough for two 1 mm spaced gels

Reagent	Volume
0.5 M Tris HCl pH 6.8	1.35 mL
dH_2O	3.15 mL
Acrylamide	500 µL
10% SDS	50 µL
10% APS	50 µL
TEMED	5 µL

The gels were assembled in the gel tank following manufacturer's instructions. Typically 20-40 µg total protein was loaded into each well of the gel alongside 2.5 µL Precision Plus Protein™ WesternC™ Standard pre-stained broad range protein standards. Samples were electrophoresed at 100 V in running buffer (25mM Tris, 192mM glycine, 0.1% SDS, pH 8.3) until the dye front reached 2 mm from the bottom of the resolving gel. The resolving gel was then either stained for total protein (Section 2.3.1.5) or transferred to a nitrocellulose membrane for Western blotting (Section 2.3.1.6).

2.3.1.5 Total protein staining

The total protein content was visualised using CooBlue MAX Protein Gel Stain, a high sensitivity (below 10 ng protein/band) stain. The gels were washed three times with 100 mL dH_2O at room temperature, for 15 min with

gentle shaking. After discarding the water, the gel was incubated with 20 mL CooBlue MAX Protein Gel Stain at 4°C overnight.

After discarding the stain, the gels were typically washed three times with 100 ml dH_2O at room temperature, for 15 min with gentle shaking. Further washes were performed if required so that clear gels with blue bands were obtained. The gels were visualised and recorded using GBox Chemi-XRQ Gel Documentation System.

2.3.1.6 Western blotting

2.3.1.6.1 Electrotransfer

After SDS-PAGE electrophoresis, the proteins were electro-transferred to nitrocellulose membranes using iBlot® 2 Dry Blotting System following manufacturer's instructions. The dry blotting was performed using a pre-programmed protocol called 'P0', a protocol designed to efficiently transfer a wide variety of proteins over a broad range of molecular weights (30-150 kDa). This protocol runs for 7 min at 20 V for 1 min, 23 V for 4 min and 25 V for the remainder.

2.3.1.6.2 Membrane blocking and antibody incubations

After transfer and disassembly of transfer pack, the nitrocellulose membrane was then incubated in 5% milk blocking buffer at room temperature, with gentle shaking for 1 h. The blocking buffer was then discarded. The membrane was washed three times with 15 mL TTBS, at room temperature with gentle shaking for 5 min per wash. The membrane was incubated with the appropriate primary antibody (Table 2.6) in 5% BSA blocking buffer at 4°C overnight.

The membrane was washed three times with 15 mL TTBS, at room temperature with gentle shaking for 5 min per wash. Membrane was incubated with secondary antibody (Table 2.6) in 5% milk blocking solution with gentle shaking at room temperature for 1 h. Precision Plus Protein™ StrepTactin-HRP Conjugate was also added into the blocking solution to detect the protein standards, typically using a 1:10,00 dilution. The membrane was washed five times with 15 mL TTBS, at room temperature with gentle shaking for 5 min per wash before proceeding to detection.

Table 2.6: Dilutions of antibodies for measurement of MAPK phosphorylation by Western blotting

	Antibody	Manufacturer	Product number	Species	Dilution
Primary	Phospho-SAPK/JNK (Thr183/Tyr185)	Cell Signaling Technology, Inc.	9251	Rabbit	1:1,000
	Phospho-c-Jun (Ser 73)		9164		
	Phospho-p38 (Thr180/Tyr182)		9215		
	Phospho-MAPKAPK-2 (Thr222)		3316		
	Phospho-p44//42 (Thr202/Tyr204)		4377		
Secondary	Anti-rabbit IgG, HRP-linked	Cell Signaling Technology, Inc.	7074	Goat	1:20,000

2.3.1.6.3 Chemiluminescent detection

SuperSignal™ West Femto Maximum Sensitivity Substrate, a chemiluminescent substrate for low-femtogram-level detection of HRP immunoblots was used. The working solution was prepared by mixing equal volumes (300 µL of each) of the Stable Peroxide Solution and the Luminol/Enhancer Solution.

The chemiluminescent working solution was applied to the membrane and incubated in the dark at room temperature for 5 min. The blot was removed from the working solution and excess reagent drained away. The chemiluminescent signal was captured using GBox Chemi-XRQ Gel Documentation System.

2.3.2 Measurement of intracellular phosphorylated MAPKs using flow cytometry

Typically, 96-well plates contained 200 µL of treated cells at a concentration of 5 x 10^5 cell/mL, along with a negative control (untreated cells) and a positive control (1 µM anisomycin, 37°C, 30 min). All cells were transferred to a V-bottomed 96-well plate, centrifuged at 500g, room temperature for 5 min and the supernatant removed.

Cells were fixed using 4% formaldehyde solution to ensure modifications, such as phosphorylation, were optimally cross-linked and cells were permeabilised using methanol to maintain structural integrity without denaturing the post translational modifications. This was achieved by resuspending the cell pellet in 100 µL 4% formaldehyde solution. Cells were incubated at 37°C for 10 min before centrifugation at 500 g, room temperature for 5 min and removal of supernatant. The cell pellet was re-suspended in 100 µL ice-cold 90 % methanol and incubated at 4°C for 30 min. If not proceeding with immunostaining immediately, cells were stored in the methanol at -20°C, overnight.

After permeabilisation, cells were centrifuged at 500 g, room temperature for 5 min and the supernatant removed. 100 µL flow cytometry incubation buffer (0.5% BSA in DPBS) was added to each well and the cells were centrifuged at 500 g, room temperature for 5 min. The supernatant was removed, and the incubation buffer wash was repeated once more. The appropriate phospho-MAPK antibody was prepared in flow cytometry incubation buffer (Table 2.7). Following the addition of 10 µL diluted antibody to each well (incubation buffer only added to unstained cells), cells were incubated in the dark, at room temperature for 1 h. 100 µL flow cytometry incubation buffer was added on top of each well to dilute any unbound antibody. The cells were centrifuged at 500 g, room temperature for 5 min and the supernatant removed. The cell pellets were re-suspended in 100 µL DPBS (without Ca^{2+} and Mg^{2+}). Stained cells were analysed immediately if possible, otherwise cells were stored in the dark at 4°C and analysed within 3 days.

Table 2.7: Antibodies for the measurement of MAPK phosphorylation by flow cytometry

Antibody	Manufacturer	Product number	Antibody dilution
Phospho-SAPK/JNK AlexaFluor® 647	Cell Signaling Technology, Inc.	9257	1: 50
Phospho- c-Jun AlexaFluor® 488		12714	
Phospho-p38 AlexaFluor® 647		4552	
Phospho-p42/44 AlexaFluor® 647		4375	

Cells were analysed on a BD Accuri C6 flow cytometer; fluidics set to medium. Viable cells only were gated (P1 in Figure 2.1). 10,000 events were collected within the viable gate and future analyses were performed only on viable cells. The negative/positive gate was set using the AlexaFluor647 histogram for the no stain control, setting the gate to include 1% of AlexaFluor647-positive cells (Figure 2.1B).

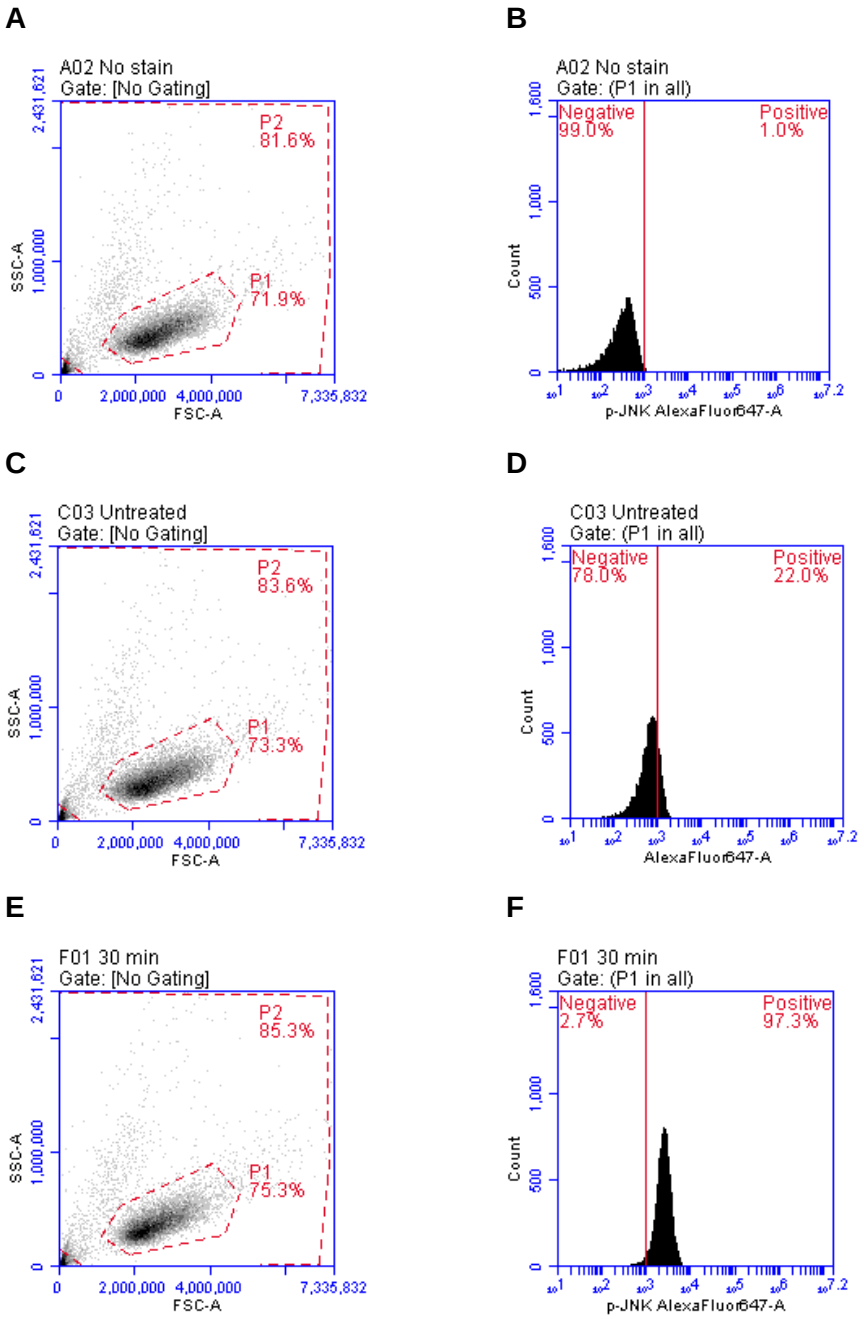

Figure 2.1: Gating strategy for measurement of MAPK phosphorylation using flow cytometry

U937 cells were treated with (A, B, C and D) 0 µM or (E,F) 1 µM anisomycin and then incubated at 37°C for 30 min. (A, C and E) show example FSC vs SSC dot plots and the gating set to only include viable cells. (B, D and F) show example histograms and the gate set to determine changes in MAPK phosphorylation.

2.4 Methods to assess cell viability

2.4.1 Plate-based assays

2.4.1.2 Preparation of dead cells for plate-based assays

Dead cells were prepared fresh prior to MTS and PI assays. U937 cells, at a concentration of 5 x 10^5 cells/mL were placed in liquid nitrogen for 1 min and then thawed at 37°C. The freeze-thaw cycle was repeated once more. Cell viability was observed microscopically; freeze-thawing the cells in liquid nitrogen caused cell death by necrosis.

2.4.1.3 Determination of metabolic activity by MTS assay

MTS assay was used to assess the metabolic activity of U937 cells. 20 µL of MTS/PES working solution (333µg/mL MTS and 25 µM PES) was added to each well containing 100 µL U937 cells. The plate was then shaken on the plate reader for 10 s and then incubated in the dark at 37°C, 5% CO_2 for 2 h. Absorbance was measured at 490 nm using Varioskan™ LUX multimode microplate reader. Metabolic activity, expressed as a percentage, was calculated by normalising the data using absorbance's for live and dead cells.

2.4.1.4 Determination of necrosis by propidium iodide staining

Propidium iodide (PI) staining was used to assess U937 cell necrosis. 100 µL PI working solution (5 µg/mL) was added to each well containing 100 µL U937 cells. The plate was shaken on the plate reader for 10 s and then the plate was incubated in the dark at room temperature for 20 min. Fluorescence intensity was measured at λ_{ex} 530 nm/ λ_{em} 645 using Varioskan™ LUX multimode microplate reader. The percentage of necrotic cells was calculated by normalising the data using absorbance's for live and dead cells.

2.4.1.5 Determination of apoptosis using caspase-3/7 fluorometric assay

Caspases 3 and 7 are effector caspases involved in apoptosis and were measured to confirm the effects of treatments on apoptosis in U937 cells. SensoLyte® Homogeneous Rh110 Caspase-3/7 Assay Kit), a fluorometric

assay, was used. The assay was performed following the kit instructions, using the microplate protocol. Kit components were thawed at room temperature prior to use. Typically, 96-well plates contained 150 µL of treated cells. A working substrate solution was prepared by adding 40 µL of 1M DTT and 10 µL caspase-3/7 substrate per ml of assay buffer. 50 µL of working substrate solution was added to each well. Reagents were mixed on plate shaker for 30 s, avoiding bubbles. The plate was incubated in the dark, at room temperature for 1 h. Fluorescence intensity was measured at λ_{ex} 496nm/ λ_{em} 520 nm using Varioskan^{TM} LUX multimode microplate reader.

2.4.2 Flow cytometry

2.4.2.1 Determination of cell viability using Annexin V & PI

Staining cells with Annexin V in combination with PI was used to distinguish between viable (annexin V-, PI -), necrotic (annexin V-, PI +), early apoptotic (annexin V+, PI -) and late apoptotic (annexin V +, PI +) cells. Cell viability was measured using FITC Annexin V FITC Apoptosis Detection Kit I. Recommended protocol for tube layout was adapted for 96-well plate as described. Typically, each well of 96-well plates contained 200 µL of treated cells or untreated cells at a concentration of 5 x 10^5 cell/mL. All cells were transferred to a V-bottomed 96-well plate, centrifuged at 500g, room temperature, for 5 min and the supernatant removed. The cell pellet was re-suspended in 100 µL ice-cold PBS and centrifuged at 500 g, room temperature for 5 min. The PBS wash was repeated once more.

The cell pellet was re-suspended in 50 µL 1 x binding buffer. 2.5 µL PI stain and 2.5 µL FITC Annexin V was added to each sample well. Compensation controls were also included: untreated, unstained cells (55 µL 1x binding buffer, no stain), untreated PI-only stained cells (52.5 µL binding buffer, 2.5 µL PI stain), untreated, annexin V-only stained cells (52.5 µL 1 x binding, 2.5 µL FITC Annexin V only). All cells were incubated in the dark, at room temperature for 15 min. 200 µL 1 x binding buffer was added on top of each well and gently mixed.

Cells were analysed immediately on BD Accuri C6 flow cytometer; fluidics set to medium. Cells were gated to exclude debris (Figure 2.2) and 10,000 events were collected within the gate. The quadrant gate, to

determine the different stages of apoptosis, was set using a PI vs Annexin V FITC plot for the no stain control (NS Ctl) (Figure 2.2B) Colour compensation was performed using individual stain controls, and applied to all the samples.

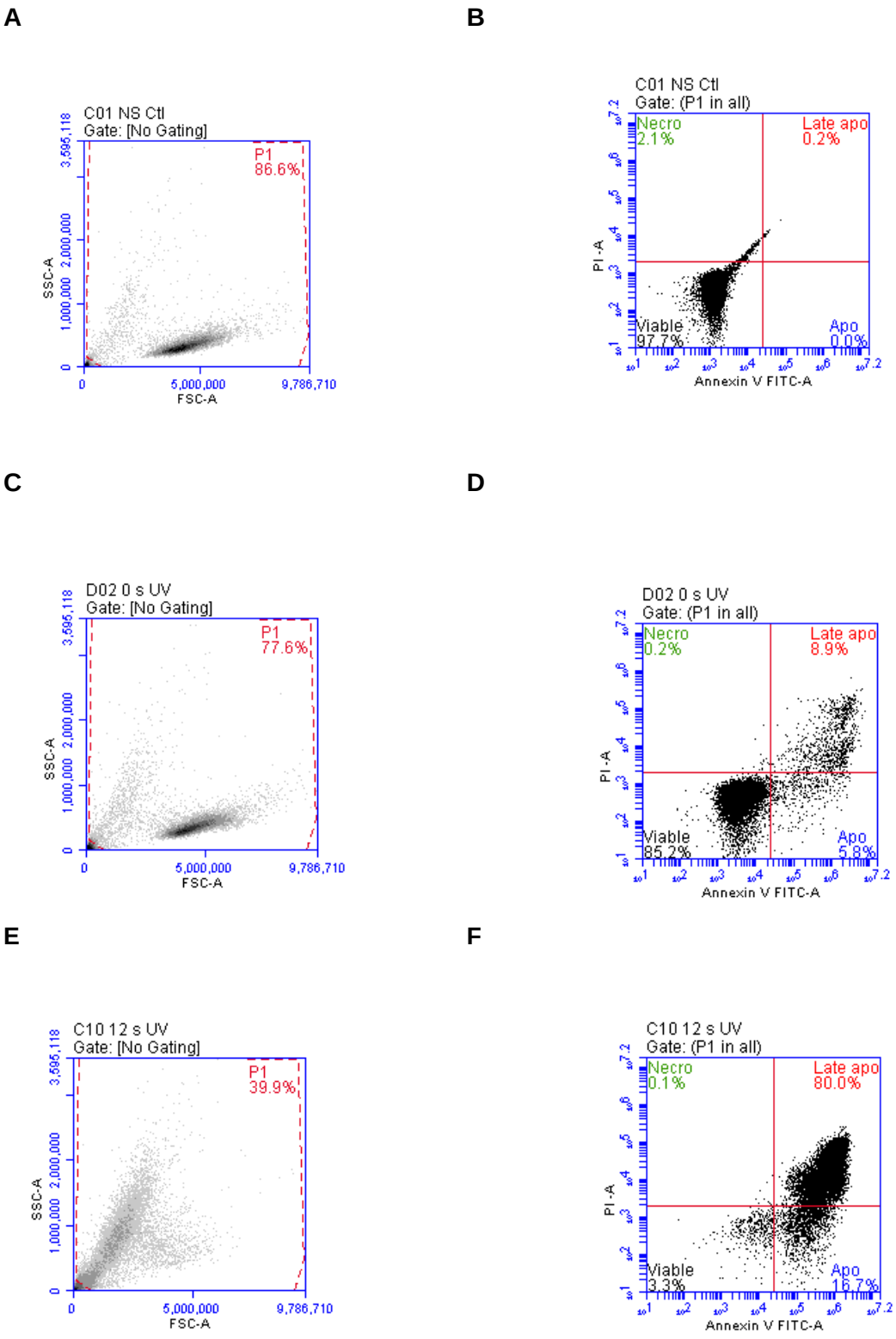

Figure 2.2: Gating strategy for FITC Annexin V Apoptosis Detection Kit
U937 cells were treated with (A, B, C and D) 0s UV or (E and F) 12 s UV and then incubated at 37°C for 24 h. Cell viability was measured using BD Pharmingen™ FITC Annexin V Apoptosis Detection Kit I. (A, C and E) show example FSC vs SSC dot plots and the gating set to exclude cell debris. (B, D and F) show example annexin V FITC vs PI dot plots and the quadrant gate set to determine the stages of cell death.

2.5 Microscopy

2.5.1 Phase-contrast microscopy

Phase-contrast microscopy was used to identify changes in morphological features of U937 cells, and hence asses the viability of U937 cells. Phase-contrast microscopy images were also collected using EVOS^{TM}FL Auto Cell Imaging System and a 40 X objective lens. Viable cells appear relatively circular in shape, as shown in Figure 2.3.

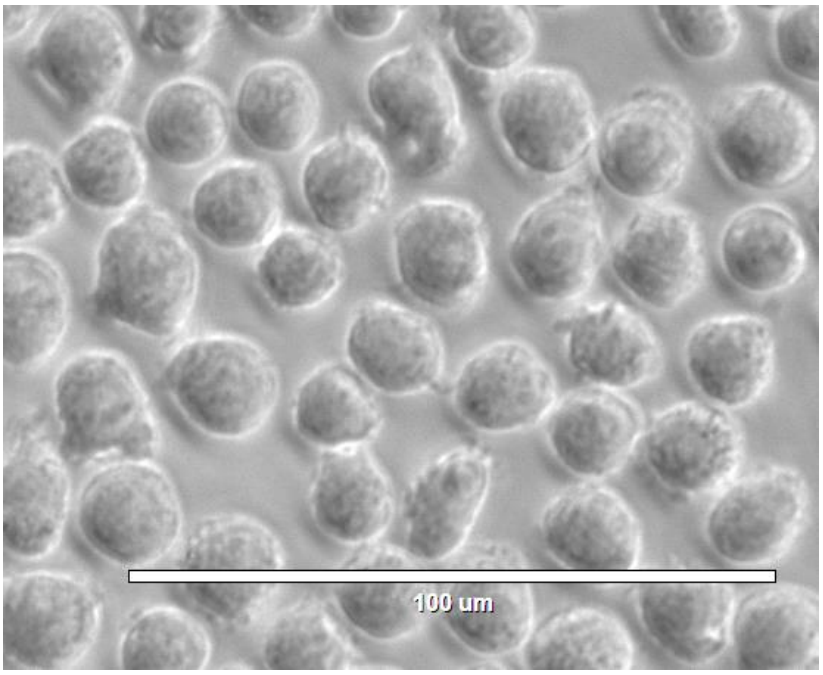

Figure 2.3: Phase-contrast microscopy image of viable U937 cells
U937 cells were visualised using EVOS^{TM}FL AutoCell Imaging System at 40X magnification. Image shown is representative of 3 individual images collected.

2.5.2 Fluorescence microscopy

2.5.2.1 Propidium iodide staining

Propidium iodide (PI) is a red-fluorescent, DNA stain which was used to identify necrotic and late apoptotic U937 cells. In viable and early apoptotic cells, cell membrane integrity excluded the PI stain (Figure 2.4). In necrotic and late apoptotic cells (dead cells), the increased membrane permeability allows the PI stain to bind to DNA, increasing the fluorescence by 20- to 30-fold. PI staining allows for the visualisation of fragmented nuclei (Figure 2.4).

2.5 µL propidium iodide staining solution (50 µg/mL) was added to 100 µL untreated and treated cells. Cells were incubated in the dark, at room temperature, for 15 min. Cells were visualised microscopically using EVOS^{TM}FL Auto Cell Imaging System. Images were collected using RFP light cube and 40 X objective lens.

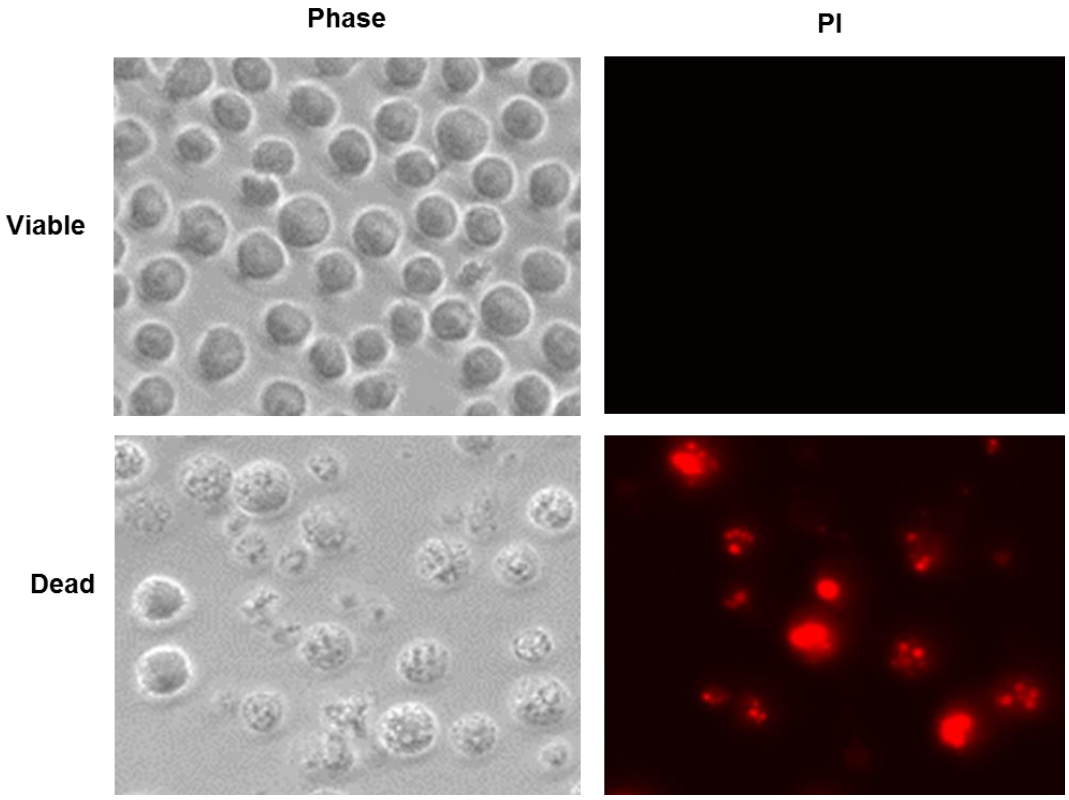

Figure 2.4: PI staining to assess cell viability
Viable (untreated) and dead (treated with 15 s UV and incubated at 37°C for 24 h) U937 cells were stained with PI staining solution and visualised using EVOS^{TM}FL AutoCell Imaging System at 40 x magnification. Images shown are representative of 3 individual images collected.

2.5.2.2 DAPI staining

DAPI (4',6-diamidino-2-phenylindole) is an alternative DNA stain that was used to visualise doxorubicin-treated cells where PI staining could not be used due to fluorescent spectral overlap. Viable cells exclude the DAPI stain, whereas for necrotic and late apoptotic cells (dead cells), where cell membrane integrity has been compromised, the nuclear material stains with DAPI (Figure 2.5).

For DAPI staining, 1 µL DAPI solution (100 µg/mL) was added to 100 µL untreated and treated cells. Cells were incubated in the dark for 5 min. Cells were visualised microscopically using EVOS^{TM} FL Auto Cell Imaging System. Images were collected using DAPI light cube and 40 X objective lens.

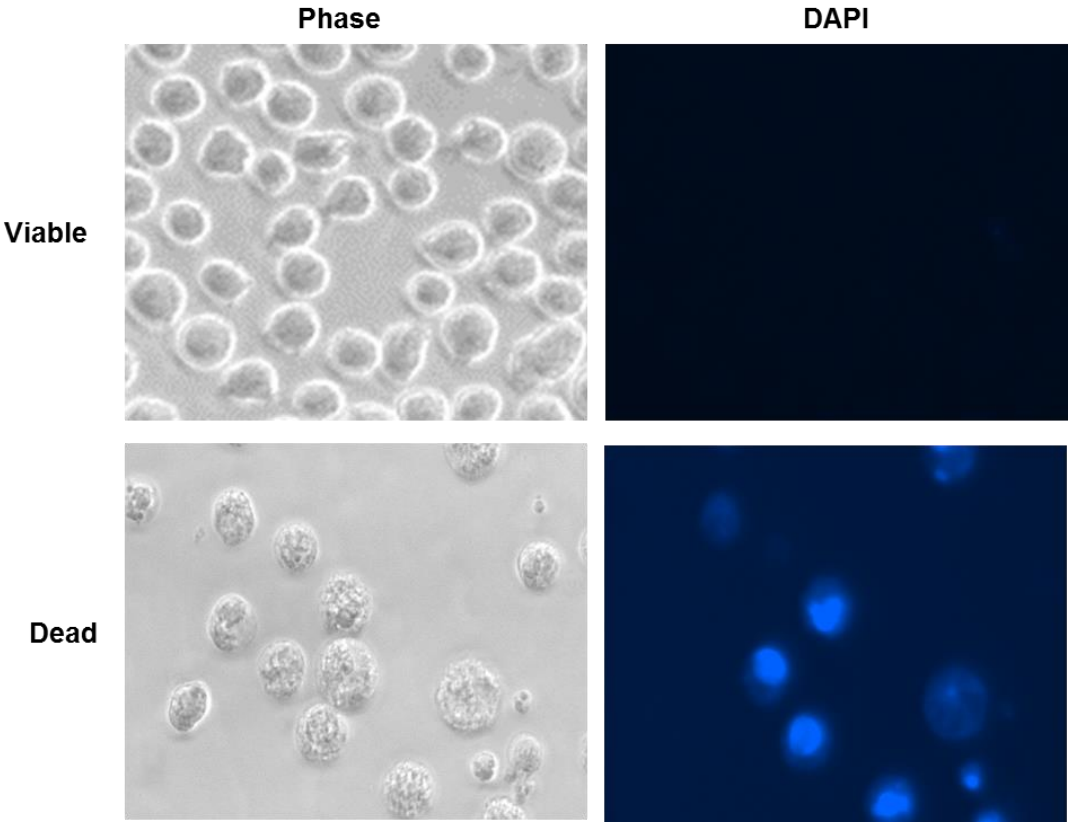

Figure 2.5: DAPI staining to assess cell viability
Viable (untreated) and dead (treated with 25 µM doxorubicin at 37°C for 24 h). U937 cells were stained with DAPI and visualised using EVOS^{TM}FL AutoCell Imaging System at 40 x magnification. Images shown are representative of 3 individual images collected.

2.6 Statistical analysis

All statistical analyses were performed using GraphPad Prism® 7.03 software. All data presented as mean ± standard deviation (SD), unless otherwise stated. Number of replicates (n) is stated in the figure legends where appropriate. Appropriate statistical tests were performed including:

- **Unpaired t-test** - used to determine statistical differences between two independent samples, for example difference between heat treatment and no heat treatment.

- **One-way ANOVA with Dunnet's *post hoc* test** – one-way ANOVA used to determine a statistical difference between the means of two or more independent groups. Dunnet's *post hoc* test used as a multiple comparison procedure to compare each of a number of treatments with a single control, for example to determine the dose-dependent effect of doxorubicin.

- **Two-way ANOVA with Sidak's *post hoc* test –** two-way ANOVA used to compare the mean difference between two groups that have been split on two independent variables. Sidaks's *post hoc* used to make a comparison between two values at each point, for example to determine the statistical difference between inhibitor and no inhibitor at mean vincristine concentration points**.**

- **Two-way ANOVA with Tukey's *post hoc* test** - Tukey's *post hoc* test is a multiple comparison test used to compare every mean with every other mean, used to make a comparison between more than two values at each point, for example to determine the difference between three concentrations of inhibitor at mean UV time points.

Chapter 3

Optimisation of the treatment of U937 cells with cell stressors

Chapter 3: Optimisation of the treatment of U937 cells with cell stressors

3.1 Introduction

Cells respond to cell stressors by altering signalling pathways, such as MAPK signalling pathways, in order to either promote cell survival or induce cell death (Simone Fulda, Gorman, Hori, & Samali, 2010). Future chapters will investigate the role of MAPK signalling pathways in U937 cell death which will be achieved by using MAPK signalling inhibitors in combination with cell stressors. Therefore in this chapter, the treatment of U937 cells with various cell stressors will be optimised.

UV light, heat treatment and chemotherapeutic agents (doxorubicin and vincristine) have been selected for investigation as the dosage of these cell stressors can be tightly controlled in an *in vitro* experimental system. UV light has previously been shown to induce apoptosis in U937 cells (Liu, You, Tashiro, Onodera, & Ikejima, 2005; Francesca Luchetti et al., 2006; Shin, Park, Yang, & Park, 2008). Although the mechanisms of action differ, both doxorubicin and vincristine are chemotherapeutic agents capable of inducing cell death in U937 cells (Ampasavate, Okonogi, & Anuchapreeda, 2010; César Ortiz-Lazareno et al., 2014; Demidenko et al., 2006; Kiburg et al., 1994). It has also been demonstrated that U937 cells are able to survive mild heat treatment (42°C) (Li, Lee, Ko, Kim, & Seo, 2000; Marfe et al., 2009) whilst lethal heat treatment (43°C and above) results in cell death (Lasunskaia et al., 2010; Li et al., 2000). Whilst the effect of these cell stressors on U937 cell death is well established, dosages of these treatments, which are effective in the experimental system used in this thesis, need to be determined.

In addition, a MAPK signalling agonist is required in future chapters in order to confirm the inhibition of MAPK signalling by selected signalling inhibitors. Anisomycin, an antibiotic which inhibits protein synthesis (Grollman, 1967), has been used in previous studies and its potential use in U937 cells will be investigated in this chapter. Anisomycin activates JNK and p38 in a range of cancer cell lines (Mauro et al., 2002; Stadheim & Kucera, 2002; Törocsik & Szeberényi, 2000) whilst the effect on ERK appears to be cell-type dependent (Bébien et al., 2003; Eriksson, Taskinen, & Leppä, 2006; Zhou et

al., 2016). Specifically in U937 cells, JNK has been reported to be activated following anisomycin treatment (Hori et al., 2008), however there is limited evidence for the effect of anisomycin on p38 and ERK phosphorylation in U937 cells. Therefore the effect of anisomycin on the JNK, p38 and ERK MAPK signalling pathways will also be investigated in this chapter.

Aims

The overall aim of this chapter is to optimise the treatment of U937 cells with cell stressors. This will be achieved by:

- studying the effects of UV light, doxorubicin, vincristine and heat treatment on U937 cell death
- determining the effects of anisomycin treatment on U937 cell death and MAPK phosphorylation

The effect of UV light, doxorubicin and vincristine on U937 cell death will firstly be assessed as it is believed these stressors will induce cell death. The effect of heat treatment will then be investigated as a stimulus to induce cell stress rather than directly inducing cell death. For all treatments, the effect on cell death will be confirmed using a range of assays. An MTS assay will provide an initial indication of the effect on cell death, by assessing metabolic activity. A PI assay will measure the percentage of PI-positive cells and hence will assess the effect on cell death by necrosis. Measuring annexin V binding and PI staining by flow cytometry and microscopy will confirm the effects of the treatments on cell death, more specifically on the stages of apoptosis.

The time-dependent effect of anisomycin on cell death will initially be assessed in order to provide an indication of the likely time period of MAPK activation. Following this, the effect of anisomycin on the phosphorylation of JNK, c-Jun, p38 and ERK will be measured by flow cytometry and Western blotting. Using two alternative techniques to measure MAPK activation will provide confidence in the results obtained.

3.2 Methods

3.2.1 Cell culturing

U937 cell line was cultured and maintained as described in Section 2.1.1. Prior to treatments, cells were prepared as described in Section 2.1.4.

3.2.2 Treatment of U937 cells with cell stressors

U937 cells were treated with the following cell stressors:

- UV light – 0 to 50 s (Section 2.2.2)
- doxorubicin – 0 to 200 µM (Section 2.2.3)
- vincristine – 0 to 200 nM (Section 2.2.3)
- heat treatment - 42°C for 1 h (Section 2.2.4)
- anisomycin (Section 2.2.5)

3.2.3 Measurement of the effects of cell stressors on cell viability

The effect of cell stressors on U937 cell viability was assessed using:

- MTS assay to measure metabolic activity (Section 2.4.1.3)
- PI assay to measure necrosis (Section 2.4.1.4)
- FITC Annexin V FITC Apoptosis Detection Kit I to measure cell viability (Section 2.4.2.1)
- SensoLyte® Homogeneous Rh110 Caspase-3/7 Assay Kit to measure active caspase 3/7 levels (Section 2.4.1.5)
- microscopy to determine changes in cell morphology (Section 2.5)

3.2.4 Measurement of the effects of anisomycin on MAPK activation

The effect of anisomycin on the phosphorylation of JNK, c-Jun, p38 and ERK was measured by:

- Western blotting (Section 2.3.1)
- flow cytometry (Section 2.3.2)

3.3 Results

3.3.1 Effect of UV light on U937 cell death

In order to examine the effect of UV light treatment on U937 cell death, U937 cells were treated with various durations of UV light and initially the effect on metabolic activity was assessed 24 h after treatment (Figure 3.1A). UV resulted in a dose-dependent reduction in metabolic activity, with metabolic activity significantly ($P<0.0001$) reduced to less than 50% following 15 s or more UV light treatment.

To determine if the observed reductions in metabolic activity were due to necrosis, a PI assay was performed (Figure 3.1B). Treatment with 15 to 50 s UV light, caused a significant ($P<0.001$) increase in the percentage of PI-positive cells compared to untreated cells. Whilst metabolic activity was reduced to 0% following treatment with 50 s UV (Figure 3.1A), only 21% of cells were PI-positive, suggesting cell death induced by UV occurs by a mechanism other than necrosis.

To identify the type of cell death induced by UV light, flow cytometry was performed to measure annexin V binding and PI staining (Figures 3.1C-E). Flow cytometry results confirmed UV light caused a significant ($P<0.05$) reduction in cell viability (Figure 3.1C). For untreated cells, cell viability was 80% (Figure 3.1C) compared to 100% metabolic activity (Figure 3.1A). This difference can be explained by the differences in the methods used, in particular with regards to the analysis. An MTS assay relies on normalisation of the data, using a live cell control, which although taken to be 100% metabolic activity, is often difficult to achieve. In comparison, flow cytometry provides a more reliable, single-cell analysis. This difference in the methods of these two assays, also explains differences between metabolic activities and viabilities observed in subsequent sections. The reductions in cell viabilities were mainly due apoptosis (Figure 3.1D). A lower percentage of cells were late apoptotic/secondary necrotic (Figure 3.1E), which accounts for the percentage of PI-positive cells observed in the PI assay (Figure 3.1B).

Microscopy images confirmed UV induced apoptosis in U937 cells (Figure 3.1F). Following UV treatment, cells were misshapen, membrane blebbing and formation of apoptotic bodies were observed, and PI staining

showed fragmented nuclei. Collectively, the results show UV light treatment induces cell death in U937 cells, by inducing apoptosis.

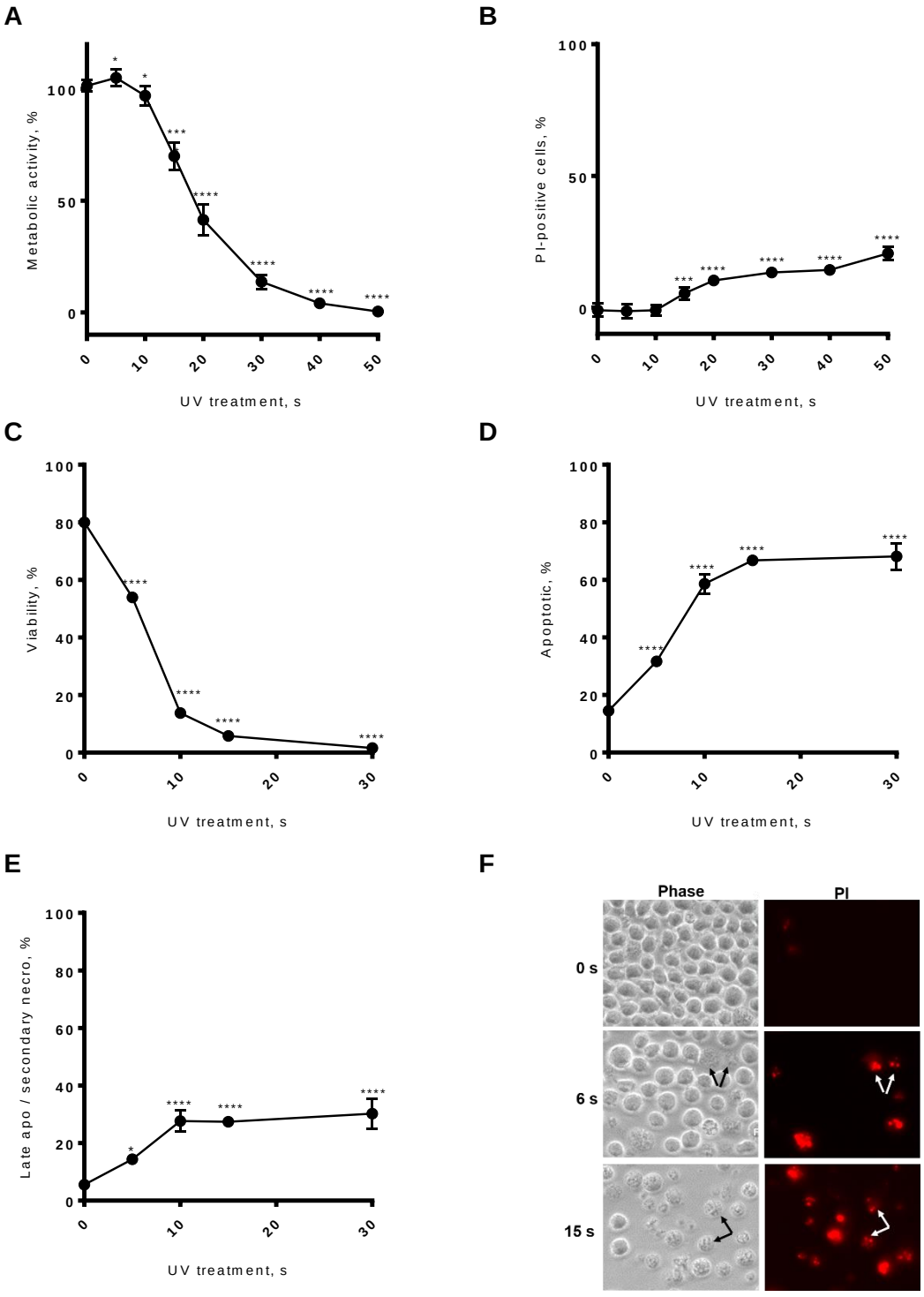

Figure 3.1: Effect of UV light on U937 cell death
U937 cells were treated with various durations of UV light and then incubated at 37°C for 24 h before an (A) MTS assay, (B) PI assay, (C-E) BD Pharmingen™ FITC Annexin V Apoptosis Detection Kit I or (F) microscopy were performed. (A-E) Data presented as mean ± SD; n=3. Statistical differences shown to untreated cells, calculated using one-way ANOVA with Dunnett's *post hoc* test: * (P<0.05), *** (P<0.001), **** (P<0.0001). (F) Cells were stained with PI staining solution and visualised using EVOS™FL AutoCell Imaging System at 40 X magnification. Arrows indicate apoptotic cells. Images shown are representative of 3 individual images collected.

3.3.2 Effect of chemotherapeutic agents on U937 cell death

3.3.2.1 Effect of doxorubicin on U937 cell death

To determine concentrations of doxorubicin which induce cell death, U937 cells were treated with a range of concentrations of doxorubicin and an MTS assay was performed (Figure 3.2A). Doxorubicin resulted in a decrease in metabolic activity in a concentration-dependent manner. Treatment with 50 µM resulted in a significant ($P<0.0001$) reduction in metabolic activity to 1%. A slight increase in metabolic activities were observed for concentrations greater than 50 µM due to the spectral overlap for doxorubicin and the MTS reagent.

As treatment with 3.13 µM to 50 µM doxorubicin resulted in reductions in metabolic activities to between 57 and 1% (Figure 3.2A), the effect of concentrations within this range, on active caspase-3/7 levels, were measured to determine if the observed reductions were specifically due to apoptosis (Figure 3.2B). Concentrations of doxorubicin greater than 5 µM resulted in a significant ($P<0.01$) increase in active caspase-3/7 levels, indicating cell death via apoptosis. Based on the data from the MTS assay (Figure 3.2A), it was expected that 5 µM doxorubicin would result in the activation of caspase-3/7, however this was not observed (Figure 3.2B). It is possible that the activation of caspase-3/7 by low concentrations of doxorubicin, takes longer than 9 h.

Microscopy images confirmed doxorubicin causes apoptosis in U937 cells (Figure 3.2C). Untreated cells appeared relatively circular and showed no DAPI fluorescence. Doxorubicin treatment increased the permeability of U937 cells to DAPI stain which showed fragmented nuclei. Collectively the results demonstrate doxorubicin induces apoptosis in U937 cells.

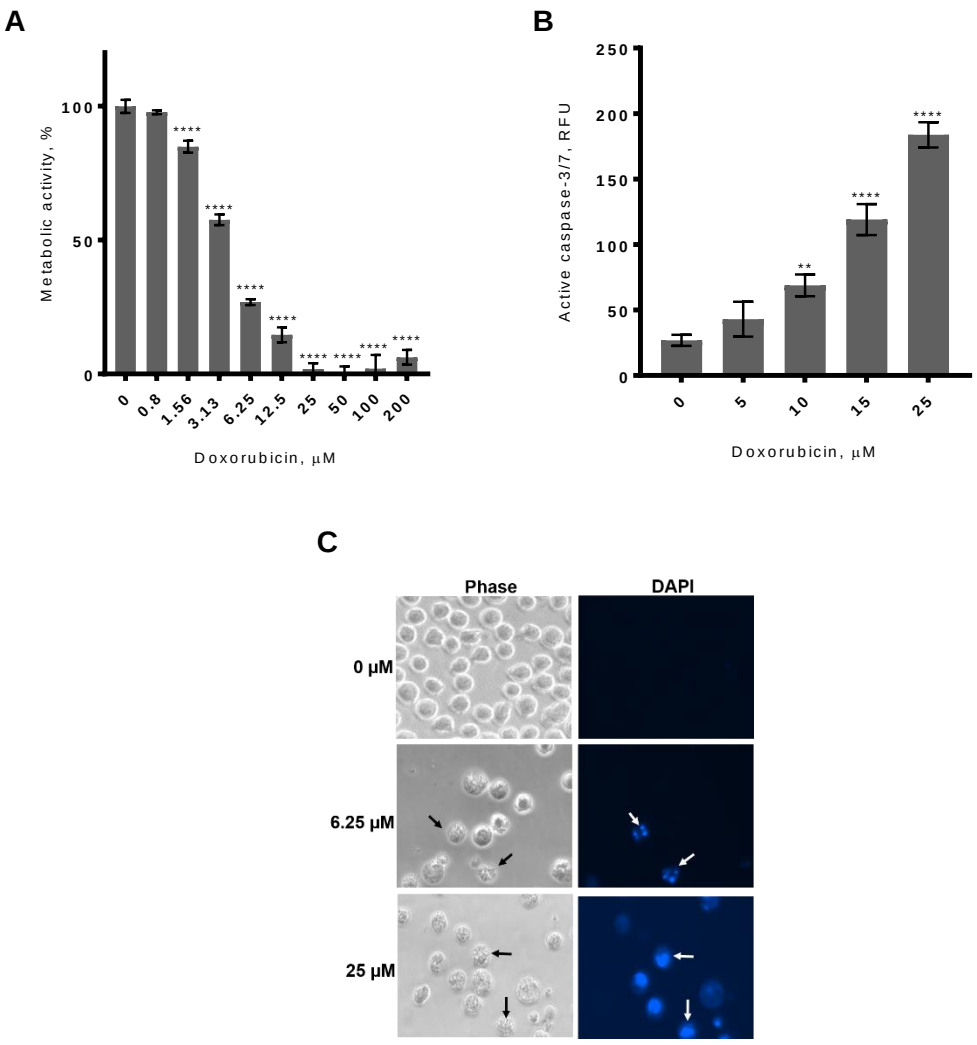

Figure 3.2: Effect of doxorubicin on U937 cell death
U937 cells were treated with various concentrations of doxorubicin and then incubated at 37°C for 24 h before (A) an MTS assay and (C) microscopy were performed or incubated for 9 h before (B) active caspase-3/7 levels were measured using SensoLyte® Homogeneous Rh110 Caspase-3/7 Assay Kit. (A, B) Data presented as mean ± SD; n=3. Statistical differences shown to untreated cells, calculated using one-way ANOVA with Dunnett's *post hoc* test: ** (P<0.01), **** (P<0.0001). (C) Cells were stained with DAPI and visualised using EVOS^{TM}FL AutoCell Imaging System at 40 x magnification. Arrows indicate apoptotic cells. Images shown are representative of 3 individual images collected.

3.3.2.2 Effect of vincristine on U937 cell death

In order to examine the effects of vincristine treatment on U937 cell death, U937 cells were treated with various concentrations of vincristine and the effect on metabolic activity was assessed 24 h after treatment (Figure 3.3A). As the concentration of vincristine increased from 0 to 0.8 nM, metabolic activities were reduced in a concentration-dependent manner (Figure 3.3A). Metabolic activity was significantly ($P<0.0001$) reduced to 44% following treatment with 0.8 nM vincristine but concentrations of vincristine greater than 0.8 nM did not cause any further reductions in metabolic activities.

To determine if the observed reductions in metabolic activities were due to necrosis, a PI assay was performed (Figure 3.3B). Although treatment with 0.8 to 200 nM vincristine resulted in a statistically significant ($P<0.01$) increase in the percentage of PI-positive cells, vincristine had little effect on necrosis. Vincristine resulted in no more than a 7% increase in PI-positive cells suggesting that the reductions in cell metabolic activities measured (Figure 3.3A) were due to cell death mechanisms other than necrosis.

To identify the type of cell death induced by vincristine, flow cytometry was performed to measure annexin V binding and PI staining (Figure 3.3C-E). Flow cytometry results confirmed reductions in cell viabilities (Figure 3.3C) were due to cell death via apoptosis (Figure 3.3D), in particular, a large percentage of cells were in the late stages of apoptosis/secondary necrosis (Figure 3.3E). Vincristine treatment had no effect on necrosis (data not shown).

Microscopy images showed the characteristic morphological features of apoptosis, such as membrane blebbing and nuclear fragmentation, confirming vincristine induces apoptosis in U937 cells (Figure 3.3F). A small number of vincristine-treated U937 cells appeared to have an altered structure, either due to the effects of microtubule disarray or the activation of U937 cells to macrophages. From the results presented, it is evident that vincristine induces apoptosis in U937 cells.

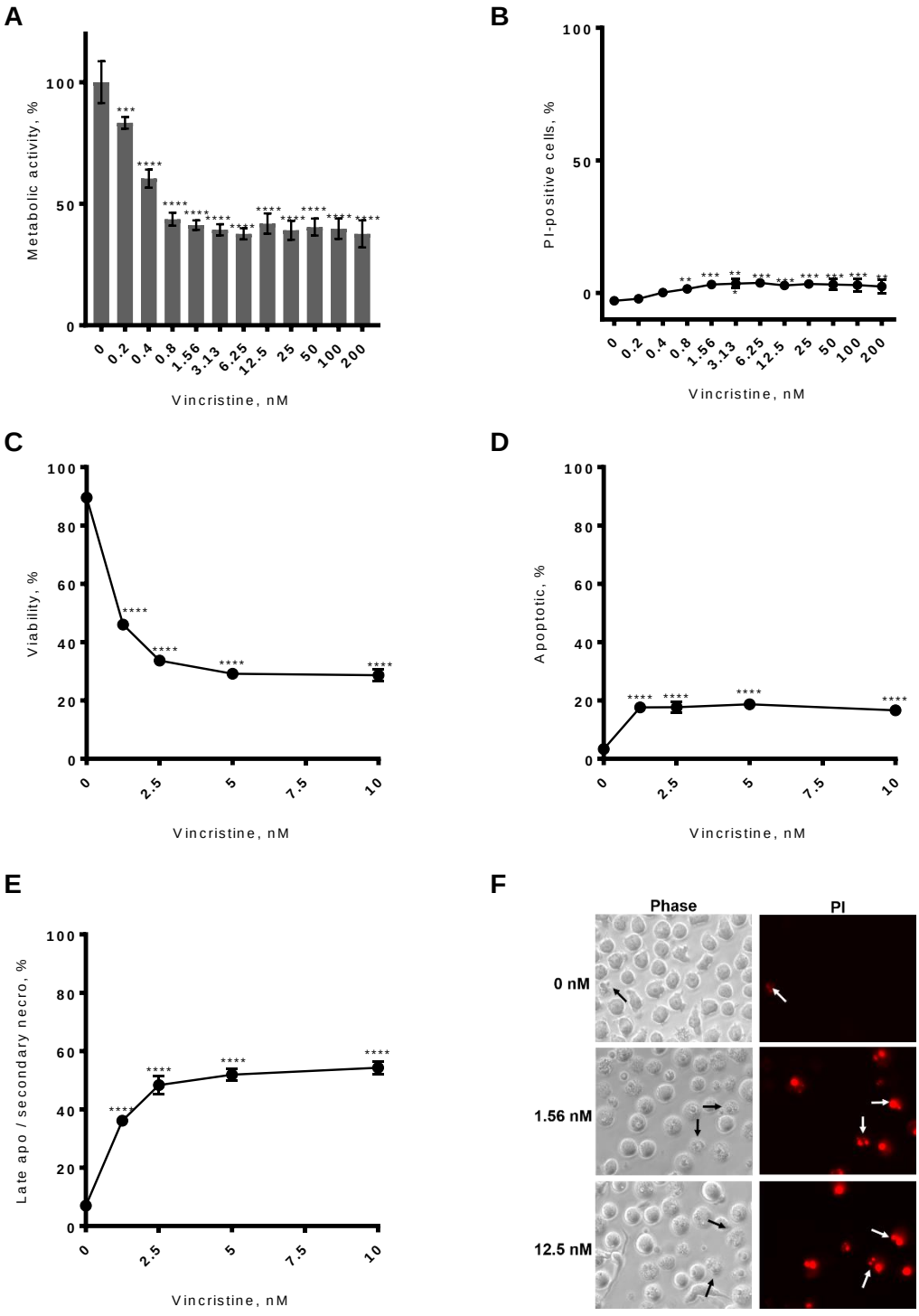

Figure 3.3: Effect of vincristine on U937 cell death
U937 cells were treated with various concentrations of vincristine for 24 h at 37°C and then incubated at 37°C for 24 h before an (A) MTS assay, (B) PI assay, (C-E) BD Pharmingen™ FITC Annexin V Apoptosis Detection Kit I or (F) microscopy were performed. (A-E) Data presented as mean ± SD; n=3. Statistical differences shown to untreated cells, calculated using one-way ANOVA with Dunnett's *post hoc* test: ** (P<0.01) *** (P<0.001), **** (P<0.0001). (F) Cells were stained with PI staining solution and visualised using EVOS™FL AutoCell Imaging System at 40 X magnification. Arrows indicate apoptotic cells. Images shown are representative of 3 individual images collected.

3.3.3 Effect of heat treatment on U937 cell death

The effect of heat treatment on U937 cell death was assessed after both 24 and 48 h. After 24 h there was a significant ($P<0.05$) decrease, by around 30%, in the metabolic activity of heat treated cells compared to non-heat treated cells (Figure 3.4A). The percentages of viable, necrotic, apoptotic and late apoptotic/ secondary necrotic cells were similar between heat treated and non-heat treated cells, 24 h after treatment (Figure 3.4B). Microscopy images showed heat treatment had no effect on U937 viability after 24 h; heat treated cells appeared morphologically similar to non-heat treated cells and neither heat treated nor non-heat treated cells stained with PI (Figure 3.4C).

After 48 h, there were no significant difference in the percentage of metabolically active cells between heat treated and non-heat treated cells (Figure 3.4D). The percentages of viable, necrotic, apoptotic and late apoptotic/ secondary necrotic cells were similar between heat treated and non-heat treated cells, 48 h after treatment (Figure 3.4E). Microscopy images confirmed heat treatment had no effect on U937 viability after 48 h (Figure 3.4F), heat treated and non-heat treated cells appeared morphologically similar 48 h after treatment; a very limited number of heat treated and non-heat treated cells stained with PI.

Collectively, these results suggest that heat treatment does not induce cell death in U937 cells. Although a decrease in metabolic activity was observed 24 h after treatment, confirmation was provided by the other tests that the reduction in metabolic activity was not due to apoptosis. It is likely that results observed were due to the effect of heat on other metabolically active cellular processes, which the cells were able to adapt and recover from after 48 h.

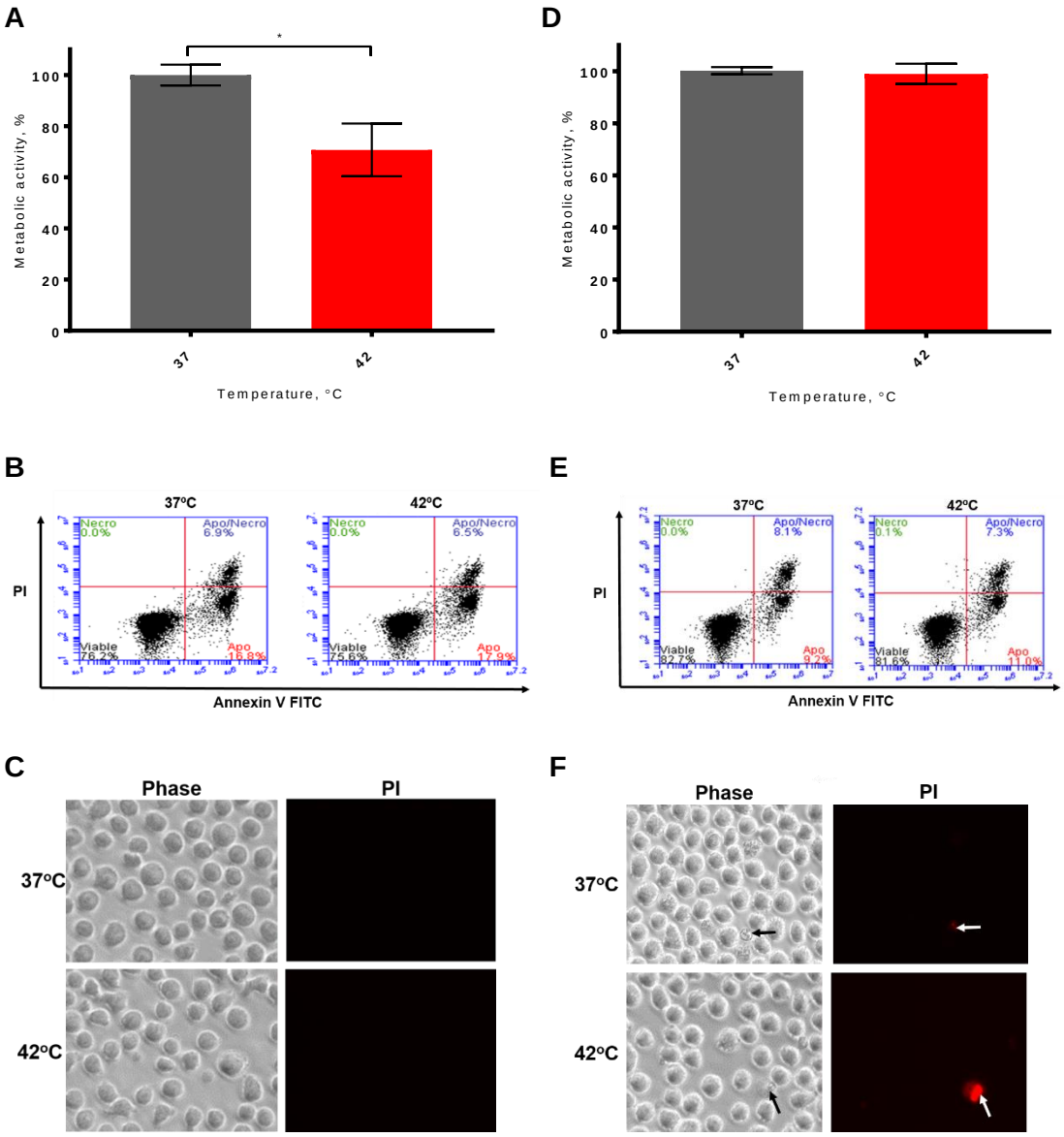

Figure 3.4: Effect of heat treatment on U937 cell death
U937 cells were incubated at either 37°C or 42°C for 1 h and then incubated at 37°C for (A,C) 24 h or (D,F) 48h before an (A,D) MTS assay, (B,E) BD Pharmingen™ FITC Annexin V Apoptosis Detection Kit I or (C-F) microscopy were performed. (A,D) Data presented as mean ± SD; n=3. Statistical differences shown to untreated cells, calculated using an unpaired t-test: * (P<0.05). (C,F) Cells were stained with PI staining solution and visualised using EVOS™FL AutoCell Imaging System at 40 X magnification. Images shown are representative of 3 individual images collected.

3.3.4 Effect of anisomycin on U937 cell death

Anisomycin was selected as a signalling agonist, to act as a positive control for the activation of MAPK signalling. A concentration of anisomycin and incubation time needed to be established for future experiments. The effect of anisomycin on cell death was initially studied, as it was believed that MAPK signalling would be activated prior to any changes in cell death being measured. U937 cells were treated with various concentrations of anisomycin and changes in metabolic activities were measured over time (Figure 3.5). Within 2 h, metabolic activities were significantly ($P<0.0001$) lower for anisomycin-treated cells compared to untreated cells, for all concentrations of anisomycin tested (Table 3.1).

U937 cells were treated with a wider range of concentrations of anisomycin and metabolic activity was assessed after 2 h (Figure 3.6). As the concentration of anisomycin increased, the metabolic activities of U937 cells decreased in a concentration-dependent manner. Metabolic activities were significantly ($P<0.0001$) reduced to less than 50% for cells treated with 1 µM or higher of anisomycin. These results indicated that MAPK signalling components are likely to be activated by 1 µM anisomycin, within 2 h of treatment.

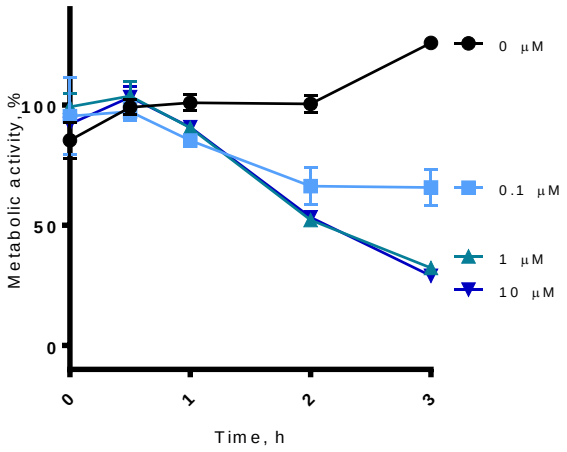

Figure 3.5: Time-dependent effect of anisomycin on U937 metabolic activity
U937 cells were treated with 0, 0.1, 1 or 10 µM concentration of anisomycin and then incubated at 37°C. MTS assays were performed after various times. Data presented as mean ± SD; n=3.

Table 3.1: Statistical analysis to show the effects of anisomycin on U937 metabolic activity
Data presented as mean ± SD; n=3. Significance shown as differences anisomycin and no treatment at time points, calculated through use of a two-way ANOVA with Tukey's *post hoc* test:** (P<0.01), **** (P<0.0001).

Anisomycin (µM)	**0 h**
0 vs 0.1	ns
0 vs 1	ns
0 vs 10	ns
Anisomycin (µM)	**0.5 h**
0 vs 0.1	ns
0 vs 1	ns
0 vs 10	ns
Anisomycin (µM)	**1 h**
0 vs 0.1	**
0 vs 1	ns
0 vs 10	ns
Anisomycin (µM)	**2 h**
0 vs 0.1	****
0 vs 1	****
0 vs 10	****
Anisomycin (µM)	**3 h**
0 vs 0.1	****
0 vs 1	****
0 vs 10	****

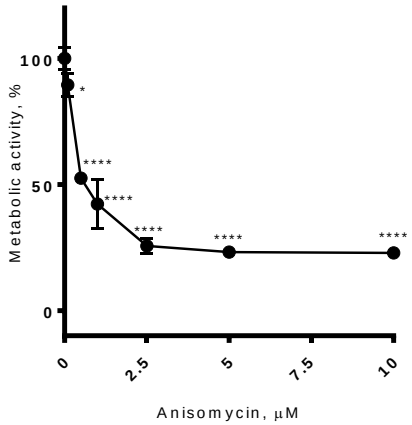

Figure 3.6: Dose dependent effect of anisomycin on U937 metabolic activity
U937 cells were treated with varying concentration of anisomycin and then incubated at 37°C for 2 h before a MTS assay was performed. Data presented as mean ± SD; n=3. Statistical differences shown to untreated cells, calculated using one-way ANOVA with Dunnett's *post hoc* test: * (P<0.05), **** (P<0.0001).

3.3.5 Effect of anisomycin on MAPK signalling components in U937 cells

Based on the data in Section 3.3.4, the dose-dependent activation of JNK following 0 to 1 µM anisomycin for 30 min was assessed (Figure 3.7). Results suggested that JNK1 and JNK2 were both phosphorylated following treatment with 0.5 and 1 µM anisomycin but JNK1/2 were not activated by lower concentrations. Whilst the bands at 46 kDa and 54 kDa indicate the presence of phospho-JNK1 and phospho-JNK2, respectively, without a positive control for phospho-JNK1/2 or a Western blot measuring total JNK, the dose-dependent effect of anisomycin on JNK phosphorylation cannot be confirmed. The effect of 1 µM anisomycin for 30 min on MAPK activation was further investigated using Western blotting and flow cytometry as a confirmatory technique.

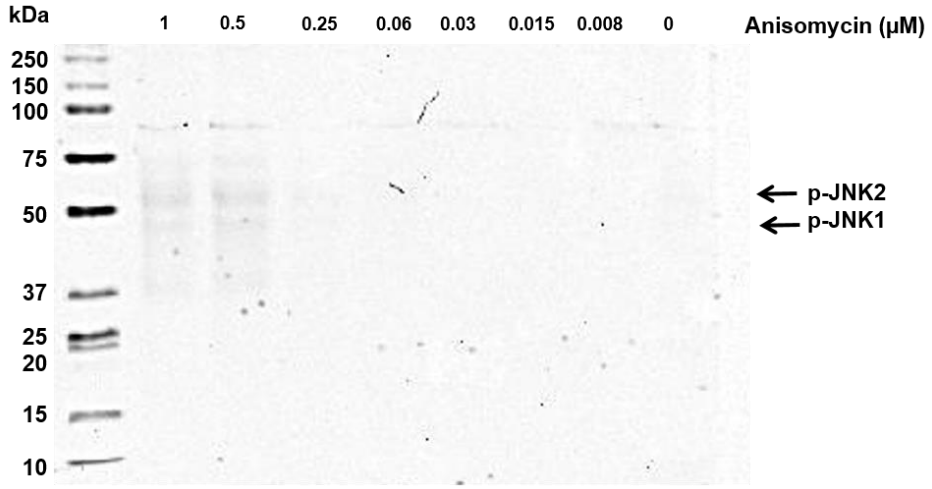

Figure 3.7: Dose dependent effect of anisomycin on JNK phosphorylation
U937 cells were treated with various concentration of anisomycin and then incubated at 37°C for 30 min. Total protein was extracted and separated with SDS-PAGE electrophoresis using 12.5% acrylamide gels. Proteins were electro-transferred to nitrocellulose membrane and phospho-JNK was detected by Western blotting using phospho-JNK/SAPK (Thr183/Tyr185) and anti-rabbit peroxidase.

Assessment of the time-dependent activation of MAPKs following anisomycin treatment showed 24% of untreated cells, expressed phosphorylated JNK (Figure 3.8A). Incubating U937 cells with 1 µM anisomycin for 0 min caused a significant increase ($P<0.01$) by 13%, in the percentage of cells expressing phosphorylated JNK compared to untreated cells. Incubation with 1 µM anisomycin for 15 min caused a further increase in the percentage of cells expressing phosphorylated JNK, to around 98%; the high levels of phosphorylation JNK were sustained after 30 and 60 min. Western blotting confirmed that JNK was activated following 15 min incubation with 1 µM anisomycin, and levels of phosphorylated JNK were sustained up to 60 min (Figure 3.8B).

Figure 3.8 highlights the importance of using multiple techniques to measure the phosphorylation of MAPK signalling components. Western blotting is a less sensitive technique than flow cytometry, as evident by the fact that 24% of untreated U937 cells expressed phosphorylated JNK (Figure 3.8A) but phosphorylated JNK was not detected in untreated cells when measured by Western blotting (Figure 3.8B). The use of Western blotting to detect MAPK signalling components, expressed at low levels, is restricted. Western blotting is however, able to detect changes in different MAPK isoforms which cannot not be achieved by the flow cytometry methods used in this study. Given the important role of different MAPK isoforms in cancer, it is important to consider the effects of treatments on each MAPK isoform.

Both techniques have limited use in the measurement of rapid activation of MAPK signalling components. Both techniques involve a processing time (the time taken for the centrifugation and wash steps between the end of the incubation time and the point of fixation or protein extraction), in which MAPKs can be activated, as demonstrated by the increase in JNK after 0 min incubation with anisomycin, compared to untreated cells (Figure 3.8A).

No difference in the percentage of cells expressing phospho c-Jun were observed between untreated cells and cells incubated with anisomycin for 0 min (Figure 3.9A). The percentage of cells expressing phosphorylated c-Jun was significantly ($P<0.0001$) increased after 30 min incubation with

anisomycin, and activation was sustained for up to 60 min. Western blotting confirmed c-Jun was activated following 15 min incubation with anisomycin (Figure 3.9B). Increased levels of phosphorylated JNK were observed after 30 min incubation with anisomycin and were sustained up to 75 min.

The percentage of cells expressing phosphorylated p38 was significantly ($P<0.0001$) higher for cells incubated with anisomycin for 0 min compared to untreated cells (Figure 3.10A). This difference can be explained by the rapid activation of p38 during the processing time, as previously discussed. The percentage of cells expressing phosphorylated p38 was further increased after 15 min incubation with anisomycin, and similar levels were observed after 30 and 60 min. Western blotting results were consistent with the flow cytometry data, confirming that p38 is rapidly activated following anisomycin treatment (Figure 3.10B).

95% of untreated U937 cells expressed phosphorylated ERK (Figure 3.11A). The percentage of cells expressing phosphorylated ERK, following treatment with anisomycin for 0 min was reduced to 87%, but this was not statistically different to untreated cells. Significant reductions in the percentages of cells expressing phosphorylated ERK were observed following incubation with anisomycin for 15 min or more. Western blotting confirmed anisomycin caused a decrease in the levels of phosphorylated ERK 15 min after treatment and continued to decrease up to 60 min (Figure 3.11B). Western blotting was unable to detect the effects of anisomycin on the different isoforms of ERK. Together, it is evident that anisomycin results in decreased levels of phosphorylated ERK.

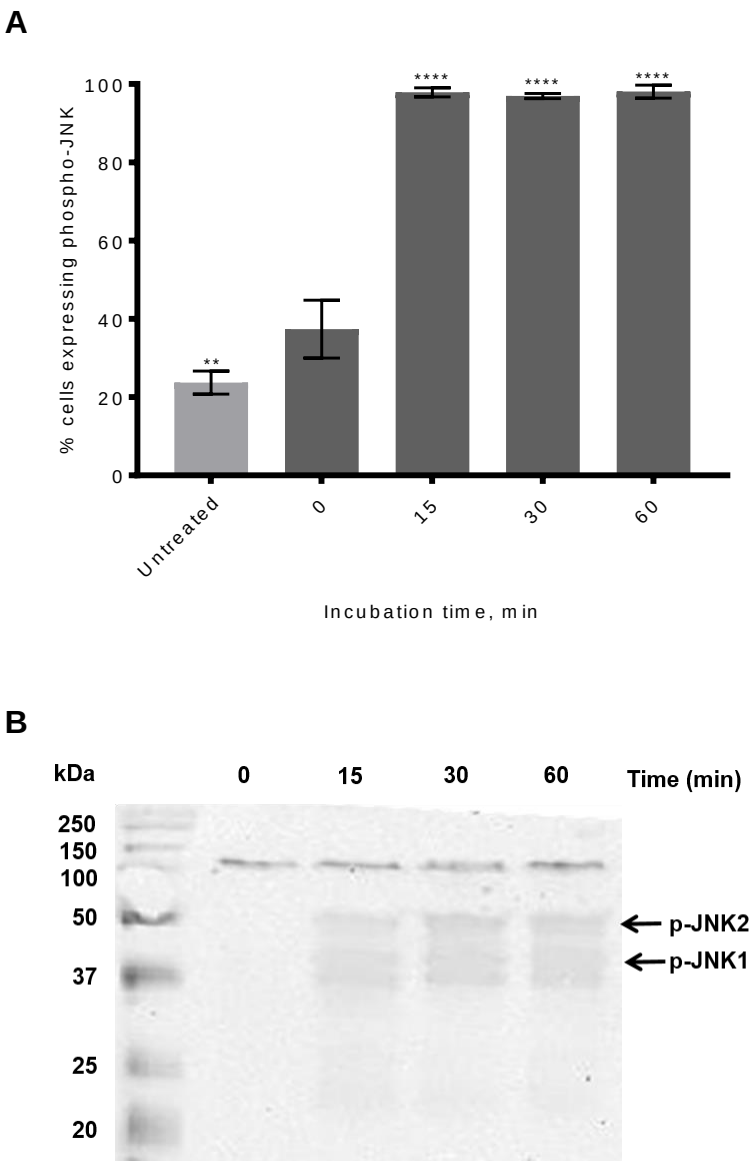

Figure 3.8: Effect of anisomycin on JNK phosphorylation
U937 cells were incubated with 1 µM anisomycin at 37°C for various times and then prepared for (A) flow cytometry or (B) Western blotting to measure JNK phosphorylation. (A) Cells were fixed with 4% paraformaldehyde, permeabilised with 90% ice-cold methanol and then stained with phospho-SAPK/JNK (Thr183/Tyr185) Alexa Fluor 647-conjugated antibody. Data presented as mean ± SD, n=3. Statistical differences shown to 0 min, calculated using one-way ANOVA with Dunnett's *post hoc* test: ** (P<0.01), **** (P<0.0001). (B) Total protein was extracted and separated with SDS-PAGE electrophoresis using 12.5% acrylamide gels. Proteins were electro-transferred to nitrocellulose membrane and phospho-JNK was detected by Western blotting using phospho-JNK/SAPK (Thr183/Tyr185) and anti-rabbit peroxidase.

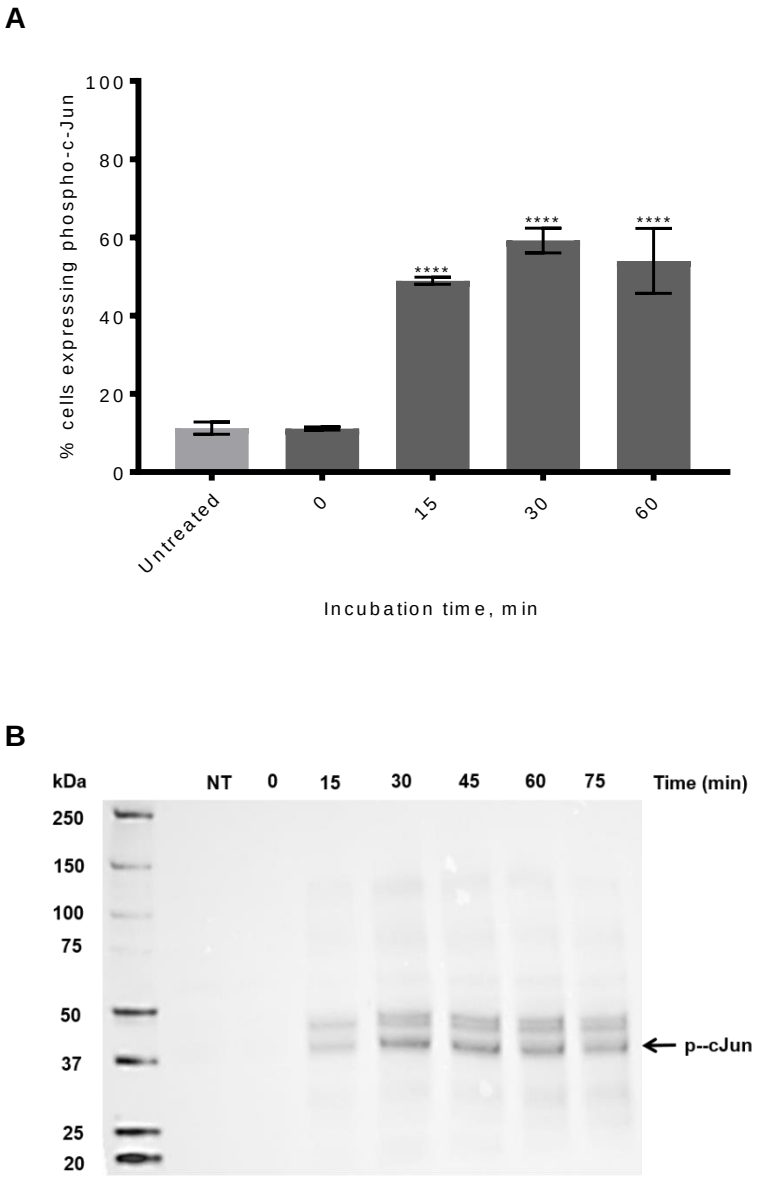

Figure 3.9: Effect of anisomycin on c-Jun phosphorylation
U937 cells were incubated with 1 µM anisomycin at 37°C for various times and then prepared for (A) flow cytometry or (B) Western blotting to measure c-Jun phosphorylation. (A) Cells were fixed with 4% paraformaldehyde, permeabilised with 90% ice-cold methanol and then stained with phospho-c-Jun (Ser73) Alexa Fluor 488-conjugated antibody. Data presented as mean ± SD, n=3. Statistical differences shown to 0 min, calculated using one-way ANOVA with Dunnett's *post hoc* test: **** (P<0.0001). (B) Total protein was extracted and separated with SDS-PAGE electrophoresis using 4-15% precast gels. Proteins were electro-transferred to nitrocellulose membrane and phospho-c-Jun was detected by Western blotting using phospho-c-Jun (Ser73) and anti-rabbit peroxidase.

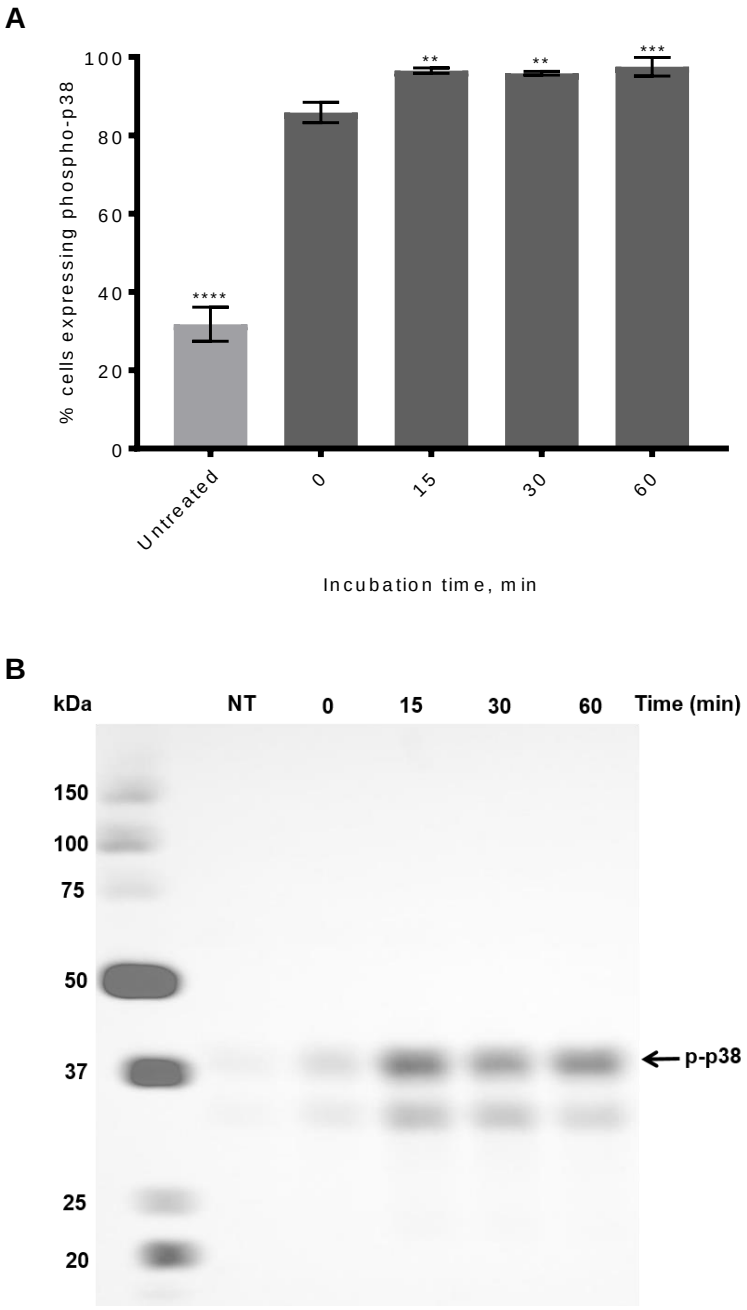

Figure 3.10: Effect of anisomycin on p38 phosphorylation
U937 cells were incubated with 1 µM anisomycin at 37°C for various times and then prepared for (A) flow cytometry or (B) Western blotting to measure p38 phosphorylation. (A) Cells were fixed with 4% paraformaldehyde, permeabilised with 90% ice-cold methanol and then stained with phospho-p38 (Thr180/Tyr182) Alexa Fluor 647-conjugated antibody. Data presented as mean ± SD, n=3. Statistical differences shown to 0 min, calculated using one-way ANOVA with Dunnett's *post hoc* test: ** (P<0.01), *** (P<0.001), **** (P<0.0001). (B) Total protein was extracted and separated with SDS-PAGE electrophoresis using 12.5% acrylamide gels. Proteins were electro-transferred to nitrocellulose membrane and phospho-p38 was detected by Western blotting using phospho-p38 (Thr180/Tyr182) and anti-rabbit peroxidase.

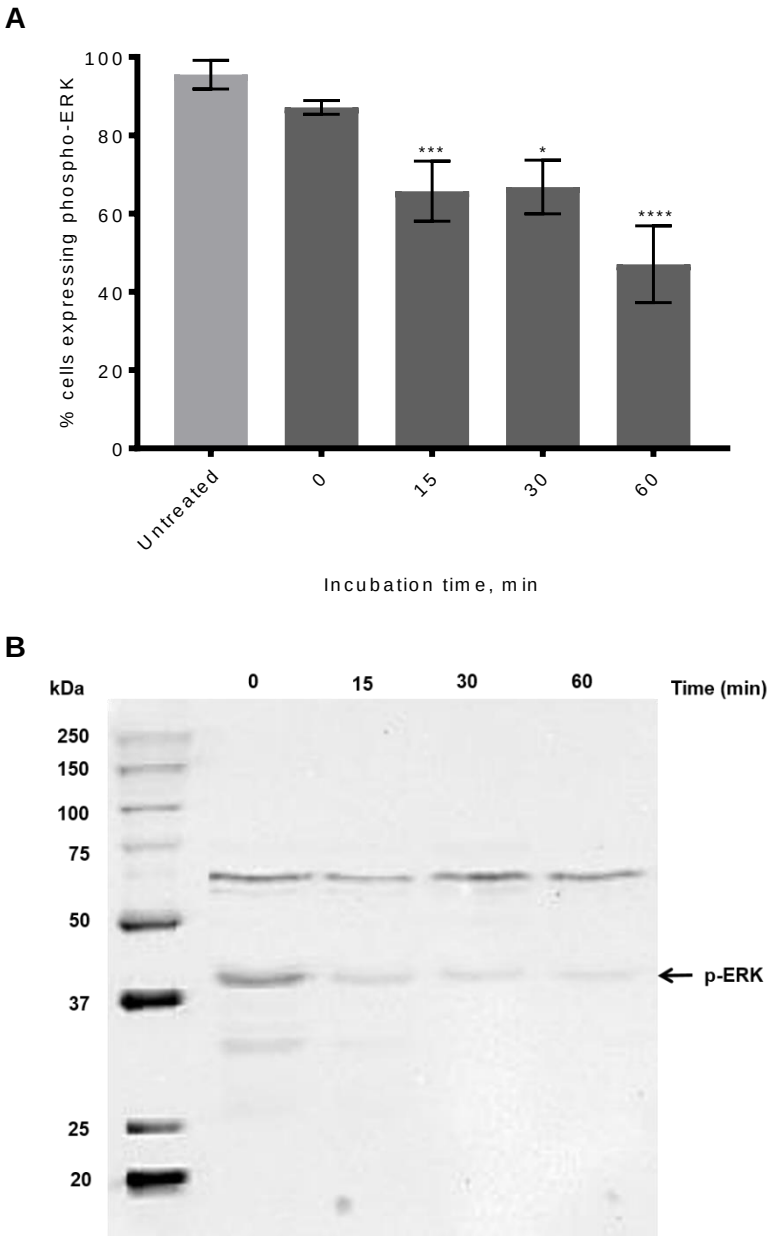

Figure 3.11: Effect of anisomycin on ERK phosphorylation
U937 cells were incubated with 1 µM anisomycin at 37°C for various times and then prepared for (A) flow cytometry or (B) Western blotting to measure ERK phosphorylation. (A) Cells were fixed with 4% paraformaldehyde, permeabilised with 90% ice-cold methanol and then stained with phospho-p44/42 (ERK1/2) (Thr202/Tyr204) Alexa Fluor 647-conjugated antibody. Data presented as mean ± SD, n=3. Statistical differences shown to 0 min, calculated using one-way ANOVA with Dunnett's *post hoc* test: * (P<0.05), *** (P<0.001), **** (P<0.0001). (B) Total protein was extracted and separated with SDS-PAGE electrophoresis using 12.5% acrylamide gels. Proteins were electro-transferred to nitrocellulose membrane and phospho-ERK was detected by Western blotting using phospho-p44/42 (Thr202/Tyr204) and anti-rabbit peroxidase.

3.4 Discussion

The overall aim of this chapter was to optimise the treatment of U937 cells with various cell stressors to include: UV light, chemotherapeutic agents (doxorubicin and vincristine), heat treatment and anisomycin.

3.4.1 UV light

The effect of UV light on U937 cell death was investigated (Section 3.3.1). UV light induced apoptosis in U937 cells in a dose-dependent manner. The apoptotic effect of UV has previously been reported in U937 cells (Liu et al., 2005; Francesca Luchetti et al., 2006; Shin et al., 2008). Different UV exposure times were tested and a range of UV times were established, which resulted in differences in cell viabilities between 0 and 100%. The main limitation of the system used was that although the duration of UV exposure could be controlled, it was not possible to measure or control the dosage of UV light. It therefore could not be ensured that the cells were treated with equivalent dosages of UV light between experiments.

3.4.2 Chemotherapeutic agents

Doxorubicin and vincristine are both chemotherapeutic agents widely used in the treatment of different types of cancer, including leukaemia. The effects of doxorubicin and vincristine on U937 cell death were investigated in this chapter (Section 3.3.2).

3.4.2.1 Doxorubicin

Doxorubicin has previously been reported to induce apoptosis in U937 cells (César Ortiz-Lazareno et al., 2014; Demidenko et al., 2006). Doxorubicin induces apoptosis in U937 cells by a mechanism involving caspases 9, 8 and 3 (César Ortiz-Lazareno et al., 2014). The results presented in this chapter are in agreement with these previous findings (Section 3.3.2.1). Doxorubicin induced cell death in a concentration-dependent manner and the decreased cell viability was attributed to apoptosis, due to the involvement of caspase-3/7 activation.

The IC_{50} for doxorubicin in this experimental system was calculated to be 3.8 µM. This differs to previously published IC_{50} values for doxorubicin on U937 cells: 0.22 µM (1 h exposure) (Hanada, Noguchi, & Yamaoka, 2006), 0.1 µM (48 h exposure) (Trendowski, Wong, Yu, & Fondy, 2015) and 0.4 µM (72 h exposure) (Hazlehurst et al., 2001). Based on the published IC_{50} values, it was predicted that the IC_{50} would be lower than 3.8 µM. A direct comparison however cannot be made between previously reported IC_{50} values and the IC_{50} value calculated in this chapter due to differences in exposure times and experimental systems. Most importantly, a concentration range of doxorubicin, which resulted in a range of metabolic activities between 0 and 100%, was established, for use in future chapters.

3.4.2.2 Vincristine

Vincristine induced cell death in U937 cells by apoptosis (Section 3.3.2.2). Metabolic activities and cell viabilities were reduced in a concentration-dependent manner following vincristine treatment. U937 cells appeared to show a degree of resistance to vincristine, as concentrations of vincristine greater than around 5 nM did not cause any further reductions in cell viabilities below 30%. It was believed 24 h was insufficient time for vincristine to have full effects on the cells. This theory is supported by the time-dependent reduction in cell growth, following vincristine treatment, in an alternative leukemic cell line, CCRF-CEM (Huschtscha, Bartierl, Ross, & Tattersall, 1996). After 24 h, growth inhibition was not decreased below 50% following treatment with high concentrations of vincristine, but after 48 and 72 h, growth was inhibited further. The IC_{50} value for vincristine in U937 cells was calculated to be 0.5 nM in this chapter. Previously reported IC_{50} values for vincristine treatment on U937 cells are: 0.1 nM (0.09 ng/mL) (48 h treatment) (Chadarat Ampasavate, Okonogi, & Anuchapreeda, 2010), 4 nM (48 h treatment) (Trendowski et al., 2015), 1.2 nM (48 h treatment) and 0.8 nM (96 h treatment) (Kiburg et al., 1994). The reporting of IC_{50} values after treatment for 48 h or more, provides further confirmation that the effects of vincristine on U937 cells are typically studied after 48 h. However, 24 h was selected as suitable for use in this thesis, as future chapters look at altering the sensitivity of U937 cells to

vincristine and the effects of MAPK signalling inhibitors were only studied after 24 h.

3.4.3 Heat treatment

Exposure of cells to heat, can induce the expression of heat shock proteins and result in protection against apoptosis (Creagh, Sheehan, & Cotter, 2000; Mosser, Caron, Bourget, Denis-Larose, & Massie, 1997). The aim here was therefore not to establish a heat treatment which induces cell death, but rather a treatment which causes minimal cell death. Heat treatments such as 43°C for 60 min or 44°C for 30 min, have been termed lethal heat treatments due to the ability of these treatments to induce apoptosis in U937 cells (Lasunskaia et al., 2010; Li et al., 2000). In contrast, previous studies have shown that mild heat treatment at 42°C for 1 h does not cause apoptosis in U937 cells (Li et al., 2000; Marfe et al., 2009). Heat treatment at 42°C for 1 h was therefore investigated in this study (Section 3.3.3).

After 24 h, heat treatment caused a decrease in metabolic activity but flow cytometry and microscopy showed heat treatment had no effect on cell viability. Heat treatment had no effect on metabolic activity or cell viability after 48 h. Collectively these results suggest that heat treatment at 42°C for 1 h has minimal effect on U937 cell death. These results are consistent with previous studies (Li et al., 2000; Marfe et al., 2009), although a study has shown that this heat treatment can induce low levels of apoptosis (less than 20%) (Samali & Cotter, 1996). These previous studies assessed the effect of heat treatment on apoptosis in U937 cells after a short recovery incubation (less than 6 h) whereas this study measured the effect on cell death after longer periods: 24 and 48 h. The main limitation is that changes in heat shock proteins following treatment were not measured, so it can only be assumed that the heat treatment used induced the expression of HSPs based on previous literature.

3.4.4 Anisomycin

Further research presented in this thesis requires the use of a signalling agonist to act as a positive control for the activation of MAPK signalling. Anisomycin was selected as a potential molecule as it is an inducer of

apoptosis and an activator of JNK and p38 signalling in several human cancer cell lines (Hori et al., 2008; Mauro et al., 2002; Stadheim & Kucera, 2002; Törocsik & Szeberényi, 2000; Zhou et al., 2016).

Anisomycin treatment induced cell death in U937 cells (Section 3.3.4). Following anisomycin treatment, reductions in metabolic activities were observed within 2 h and 1 µM anisomycin was sufficient to reduce metabolic activity to less than 50% after 24 h. These results are in agreement with another study utilising U937 cells (Hori et al., 2008). Involvement of the apoptotic mitochondrial caspase pathway is required for anisomycin-induced cell death in U937 cells (Hori et al., 2008).

The dose-dependent and time-dependent activation of JNK was assessed in U937 cells following anisomycin treatment (Section 3.3.5). Treatment with 1 µM anisomycin for 15 min was sufficient to activate JNK and activation was sustained up to 60 min. The observed effects of anisomycin on JNK activation in U937 cells are consistent with the findings of a previous study (Hori et al., 2008), but the effects of anisomycin on c-Jun, p38 and ERK were not investigated by Hori et al. The results presented here show c-Jun was activated to a certain extent 15 min after anisomycin treatment but the levels of phosphorylated c-Jun were increased further after 30 min. The delayed phosphorylation of c-Jun, compared to JNK, can be expected as c-Jun is a downstream target of JNK. Anisomycin caused a rapid activation in p38, with phosphorylated p38 levels being increased immediately after anisomycin treatment and being sustained up to 60 min. Taken together, these results suggest that treatment with 1 µM anisomycin for 30 min, is the most suitable concentration and incubation time to use to induce the activation of JNK and p38 in combination with MAPK inhibitors in Chapters 4 and 5.

High levels of phosphorylated ERK were measured in untreated U937 cells, which can be expected given the important role of ERK cell survival, differentiation and proliferation. Whilst it is well established that anisomycin activates JNK and p38 in a wide range of cancer cell lines, (Hori et al., 2008; Mauro et al., 2002; Stadheim & Kucera, 2002; Törocsik & Szeberényi, 2000; Zhou et al., 2016) the effect on ERK remains unclear and appears to be cell-specific. ERK signalling is unaffected by anisomycin treatment in Jurkat cells

(Zhou et al., 2016). Following anisomycin treatment, ERK is weakly activated in PC12 cells (Eriksson et al., 2006) and strongly activated in HOS cells (Bébien et al., 2003). The results of this chapter showed anisomycin inhibits the phosphorylation of ERK in U937 cells.

3.5 Summary

Collectively, the work presented in this chapter shows that UV light, doxorubicin, vincristine and anisomycin are able to induce cell death in U937 cells whereas heat treatment does not. In addition, anisomycin activates components of the JNK and p38 signalling pathways but inhibits ERK signalling. The following chapters will investigate the role of MAPK signalling pathways in response to the cell stressors optimised in this chapter. Based on the results of this chapter, the following doses of cell stressors will be used in Chapters 4, 5 and 6:

- UV treatment - 0 to 50 s
- doxorubicin - 0 to 50 µM
- vincristine - 0 to 100 nM
- heat treatment - 42°C for 1 h
- anisomycin - 1 µM for 30 min

Chapter 4

Targeting JNK signalling in U937 cell line

Chapter 4: Targeting JNK signalling in U937 cell line

4.1 Introduction

The previous chapter demonstrated the effects of various cell stressors on U937 cell death. In this chapter, the c-Jun N-terminal kinase (JNK) signalling pathway is targeted, in order to understand the role of JNK signalling in the response of U937 cells to these stressors.

The JNK signalling pathway is typically activated by stress stimuli and is involved in mediating both cell survival and cell death (Bubici & Papa, 2014). The role of JNK signalling in the development and progression of solid cancers is well-established, with evidence JNK can act as both a tumour promoter and a tumour suppressor (Tournier, 2013). The role of JNK signalling in leukemogenesis however remains poorly understood. There is limited evidence which suggests JNK signalling is involved in the development of chronic myeloid leukaemia; the constitutive activation of JNK in CML cells is essential for the transforming ability and survival of BCR/ABL-expressing cells (Dickens et al., 1997; Hess et al., 2002; Raitano et al., 1995). The role of JNK signalling in the development and survival of other types of leukaemia however remains unclear. Further research is required to establish the role of JNK signalling in mediating cell survival in alternative types of leukaemia, including AML. This chapter will therefore investigate the role of JNK signalling in U937 cell death, by targeting JNK signalling in combination with the cell stressors: UV light and heat treatment.

Activation of JNK signalling in leukemic cells is a common event following chemotherapy treatment (Bates, Salerni, Lowrey, & Eastman, 2011; Davison et al., 2004; Peng et al., 2016). Previous studies have shown JNK activation is essential for cell death induced by anti-leukemic agents, such as ceramide and PBOX-6 (Mc Gee et al., 2002; Nica et al., 2008). Failure to activate JNK signalling in leukemic cells may therefore be a possible mechanism of chemoresistance, which has been shown to be the case for the resistance of AML cells to daunorubicin (Lagadinou et al., 2008). Previous studies have suggested high levels of activated JNK could provide an alternative mechanism of chemoresistance in leukemic cells (Cripe et al., 2002; Leung et al., 2008; Liou et al., 2017). The role of JNK signalling in mediating

chemoresistance therefore appears to be cell- and agent- specific. Gaining a greater understanding of the role of JNK signalling in response to specific chemotherapeutic agents will aid future therapies, by understanding how these pathways can be targeted in order to reduce chemoresistance. In this chapter the role of JNK signalling in doxorubicin and vincristine-induced cell death in U937 cells will be investigated.

Aims

The overall aim of this chapter is to gain a greater understanding of the role of JNK signalling in leukemic cell death using the model U937 cell line. This will be achieved by:

- targeting JNK signalling using the chemical inhibitors SP600125 and JNK-IN-8;
- determining the effects of JNK signalling inhibition on the susceptibility of U937 cells to various cell stressors (UV light, heat and chemotherapeutic agents).

Targeting JNK signalling using two different inhibitors will allow for a more thorough understanding of the role of JNK signalling and will provide confidence in the results obtained. Prior to using the JNK inhibitors in combination with cell stressors, the effect of each inhibitor on cell death and its ability to inhibit JNK signalling must be tested. A concentration of each inhibitor, which results in inhibition of JNK signalling, but causes minimal cell death, needs to be established. The ability of each inhibitor to inhibit JNK signalling will be investigated using two alternative techniques: flow cytometry and Western blotting. The effect of each inhibitor on cell death will be studied using multiple techniques including: MTS assay, PI assay and flow cytometry to measure annexin V binding and PI staining. These techniques to assess cell death, in addition to caspase-3/7 assay and microscopy, will be used to confirm the effects of JNK inhibition and cell stressors on U937 cell death.

4.2 Methods

4.2.1 Cell culturing

U937 cell line was cultured and maintained as described in Section 2.1.1. Prior to treatments, cells were prepared as described in Section 2.1.4.

4.2.2 Treatment of U937 cells with JNK inhibitors

The JNK inhibitors, SP600125 and JNK-IN-8, prepared as described in Section 2.2.1, were used in this chapter. U937 cells were treated with SP600125 and JNK-IN-8 as described in Section 2.2.1.

4.2.3 Measurement of the effects of JNK inhibition on U937 cell death

The effect of JNK inhibition on U937 cell death was assessed using:

- MTS assay to measure metabolic activity (Section 2.4.1.3)
- PI assay to measure necrosis (Section 2.4.1.4)

4.2.4 Treatment of U937 cells with JNK inhibitor and anisomycin

U937 cells were treated with the JNK inhibitors and anisomycin as described in Section 2.2.

4.2.5 Measurement of the effects of JNK inhibition on MAPK activation

The effect of JNK inhibition and anisomycin on the phosphorylation of JNK and c-Jun was measured by

- Western blotting (Section 2.3.1)
- flow cytometry (Section 2.3.2)

4.2.6 Treatment of U937 cells with JNK inhibitors and cell stressors

U937 cells were treated with the JNK inhibitors in combination with the cell stressors, as described in Section 2.2.

4.2.7 Measurement of the effects of JNK inhibition and cell stressors on U937 cell death

The effect of JNK inhibition and cell stressors on U937 cell viability was assessed using:

- MTS assay to measure metabolic activity (Section 2.4.1.3)
- PI assay to measure necrosis (Section 2.4.1.4)

- FITC Annexin V FITC Apoptosis Detection Kit I to measure cell viability (Section 2.4.2.1)
- SensoLyte® Homogeneous Rh110 Caspase-3/7 Assay Kit to measure active caspase 3/7 levels (Section 2.4.1.5)
- microscopy to determine changes in cell morphology (Section 2.5)

4.3 Results

4.3.1 Inhibition of JNK signalling

4.3.1.1 Effect of SP600125 on U937 cell death

Initially the dose-dependent effect of SP600125 on U937 cell death was investigated. As the concentration of SP600125 increased, metabolic activity was reduced in a concentration-dependent manner (Figure 4.1A). A DMSO vehicle control confirmed that the observed effects were due to SP600125 and not the DMSO carrier (data not shown). To establish if the observed reductions in metabolic activity were due to cell death, the effect on necrosis and apoptosis was investigated using a PI assay and flow cytometry to measure annexin V binding and PI staining. Treatment with SP600125 had no effect on necrosis in U937 cells (data not shown).

Cell viability was significantly reduced for cells treated with 10 µM ($P<0.01$) and 25 µM SP600125 ($P<0.05$) (Figure 4.1B). Although cell viability was significantly reduced, it was only reduced by 22% for cells treated with 10 µM SP600125. The observed differences in cell viabilities were due to cells both in the early and late stages of apoptosis (Figure 4.1C & 4.1D). For similar concentrations of SP600125, greater reductions in metabolic activities were measured (Figure 4.1A) compared to the changes in cell viabilities (Figure 4.1B), which suggests the reductions in metabolic activities were due to the ability of SP600125 to affect cellular processes other than cell death. Collectively, these results suggest that although concentrations of SP600125 up to 25 µM do cause some cell death in U937 cells, the effect is minimal.

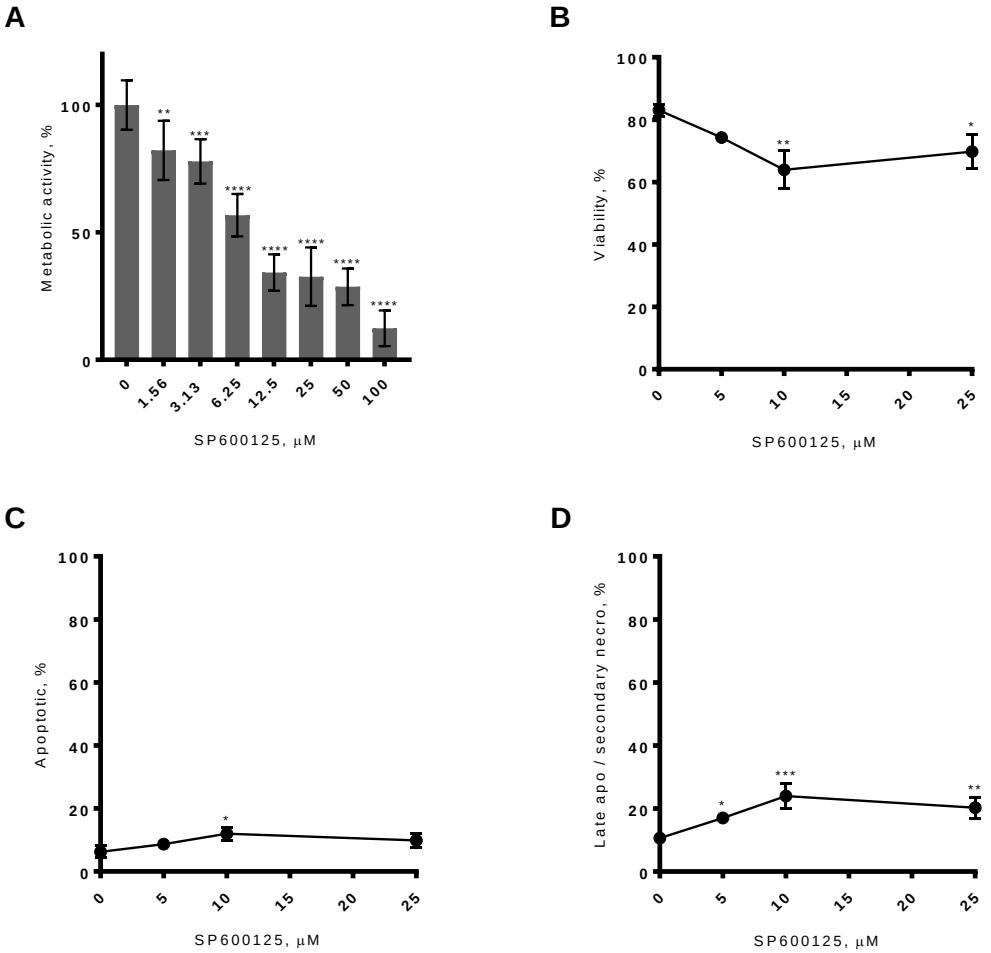

Figure 4.1: Effect of SP600125 treatment on U937 cell death
U937 cells were treated with various concentrations of SP600125 and then incubated at 37°C for 24 h before an (A) MTS assay or (C-E) BD Pharmingen™ FITC Annexin V Apoptosis Detection Kit I were performed. Data presented as mean ± SD; n=3. Statistical differences shown to untreated cells, calculated using one-way ANOVA with Dunnett's *post hoc* test: * (P<0.05), ** (P<0.01), *** (P<0.001), **** (P<0.0001).

4.3.1.2 Effect of SP600125 on MAPK phosphorylation

Based on the viability data in the previous section, the ability of 0 to 25 µM SP600125 to inhibit JNK signalling was measured by flow cytometry and Western blotting. Phosphorylation of JNK was significantly ($P<0.0001$) induced following anisomycin treatment; pre-treatment with SP600125 had no effect on the percentage of cells expressing phosphorylated JNK (Figure 4.2A). Measurement of Alexa Fluor 647 mean fluorescence intensity (MFI) showed pre-treatment with 5 to 25 µM SP600125 caused a decrease in the MFI compared to anisomycin-untreated cells but this was only significantly ($P<0.05$) lower for cells pre-treated with 25 µM SP600125 (Figure 4.2B). Western blotting however indicated that JNK phosphorylation was partially inhibited by 10 and 25 µM SP600125 (Figure 4.2C). Whilst total protein staining (data not shown) indicated equal total protein loading, the results of the Western blotting are limited due to the lack of a total-JNK Western blot.

Due to the minimal effects of SP600125 on JNK phosphorylation, further confirmation was required to confirm the ability of SP600125 to inhibit JNK signalling. The effect of SP600125 on c-Jun phosphorylation, a downstream target of JNK, was measured by flow cytometry. The percentages of cells expressing phosphorylated c-Jun were significantly ($P<0.0001$) lower for cells pre-treated with SP600125 compared to anisomycin-only treated cells (Figure 4.2D); the mean fluorescence intensity showed similar patterns of change (Figure 4.2E). These results show that although SP600125 has minimal effects on JNK phosphorylation *per se*, inhibition of JNK signalling occurs, as evidenced from the inhibition of c-Jun phosphorylation. Whilst the percentage of cells expressing phosphorylated c-Jun was significantly ($P<0.0001$) reduced for cells pre-treated with 5 µM, the effect was minimal and was greater for 10 µM, hence 10 µM SP600125 or higher was used in future experiments.

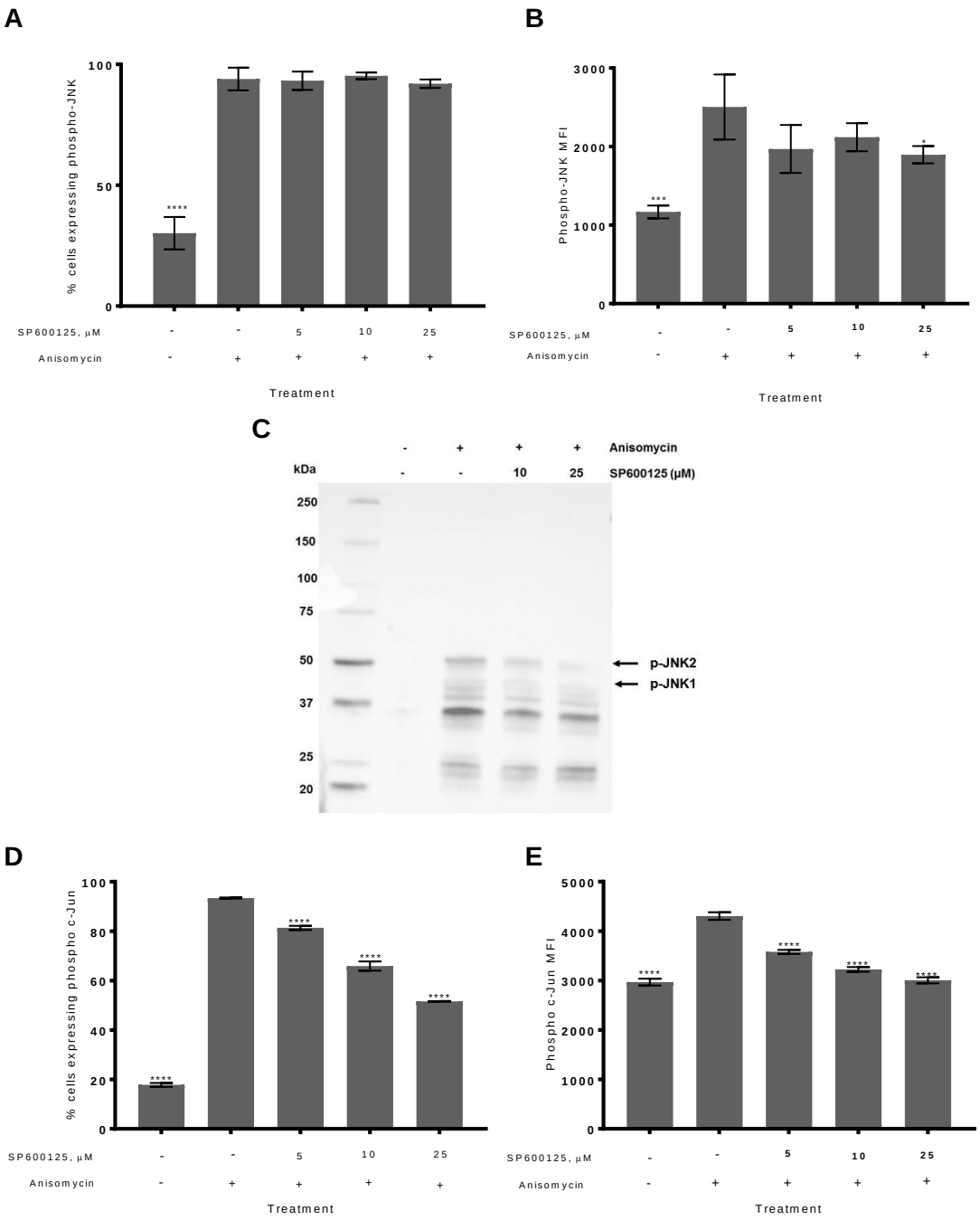

Figure 4.2: Effect of SP600125 and anisomycin on JNK and c-Jun phosphorylation

U937 cells were pre-treated with various concentrations of SP600125 at 37°C for 1 h and then incubated with 1 µM anisomycin at 37°C for 30 min. Cells were then prepared for (A,B,D,E) flow cytometry or (C) Western blotting. (A,B,D,E) Cells were fixed with 4% paraformaldehyde, permeabilised with 90% ice-cold methanol and stained with (A,B) phospho-SAPK/JNK (Thr183/Tyr185) Alexa Fluor 647-conjugated antibody or (D,E) phospho-c-Jun (Ser73) Alexa Fluor 488-conjugated antibody. (A,D) shows the percentage of cells expressing phospho-JNK/c-Jun and (B,E) shows phospho-JNK/c-Jun mean fluorescence intensity (MFI). Data presented as mean ± SD, n=3. Statistical differences shown to anisomycin-only treated cells, calculated using one-way ANOVA with Dunnett's *post hoc* test: * (P<0.05), *** (P<0.001), **** (P<0.0001). (C) Total protein was extracted and separated with SDS-PAGE electrophoresis using 4-15% precast gels. Proteins were electro-transferred to nitrocellulose membrane and phospho-JNK was detected by Western blotting using phospho-JNK/SAPK (Thr183/Tyr185) and anti-rabbit peroxidase.

4.3.1.3 Effect of JNK-IN-8 on U937 cell death

Assessment of the dose-dependent effect of JNK-IN-8 on U937 metabolic activity showed that treatment with 0.05 to 6.25 µM JNK-IN-8 caused significant ($P<0.01$) increases in metabolic activities compared to untreated cells (Figure 4.3A). Treatment with greater than 12.5 µM JNK-IN-8 caused a significant ($P<0.0001$) decrease in metabolic activity compared to untreated cells. A DMSO vehicle control confirmed that the observed effects were due to JNK-IN-8 and not the DMSO carrier (data not shown). Treatment with up to 25 µM JNK-IN-8 had no effect on U937 necrosis, however necrosis was observed by the PI assay (Figure 3.4B) and flow cytometry (Figure 4.3D) for 25 µM of JNK-IN-8 or higher.

The effects of concentrations of JNK-IN-8 between 1 and 25 µM on U937 cell death were investigated further using flow cytometry (Figure 4.3C-F). Treatment with 1.0, 2.5 and 5.0 µM JNK-IN-8 had minimal effect on U937 viability (Figure 4.3C). Reduced cell viabilities were measured for cells treated with higher concentrations of JNK-IN-8, due to cells in the late stage of apoptosis (Figure 4.3F).

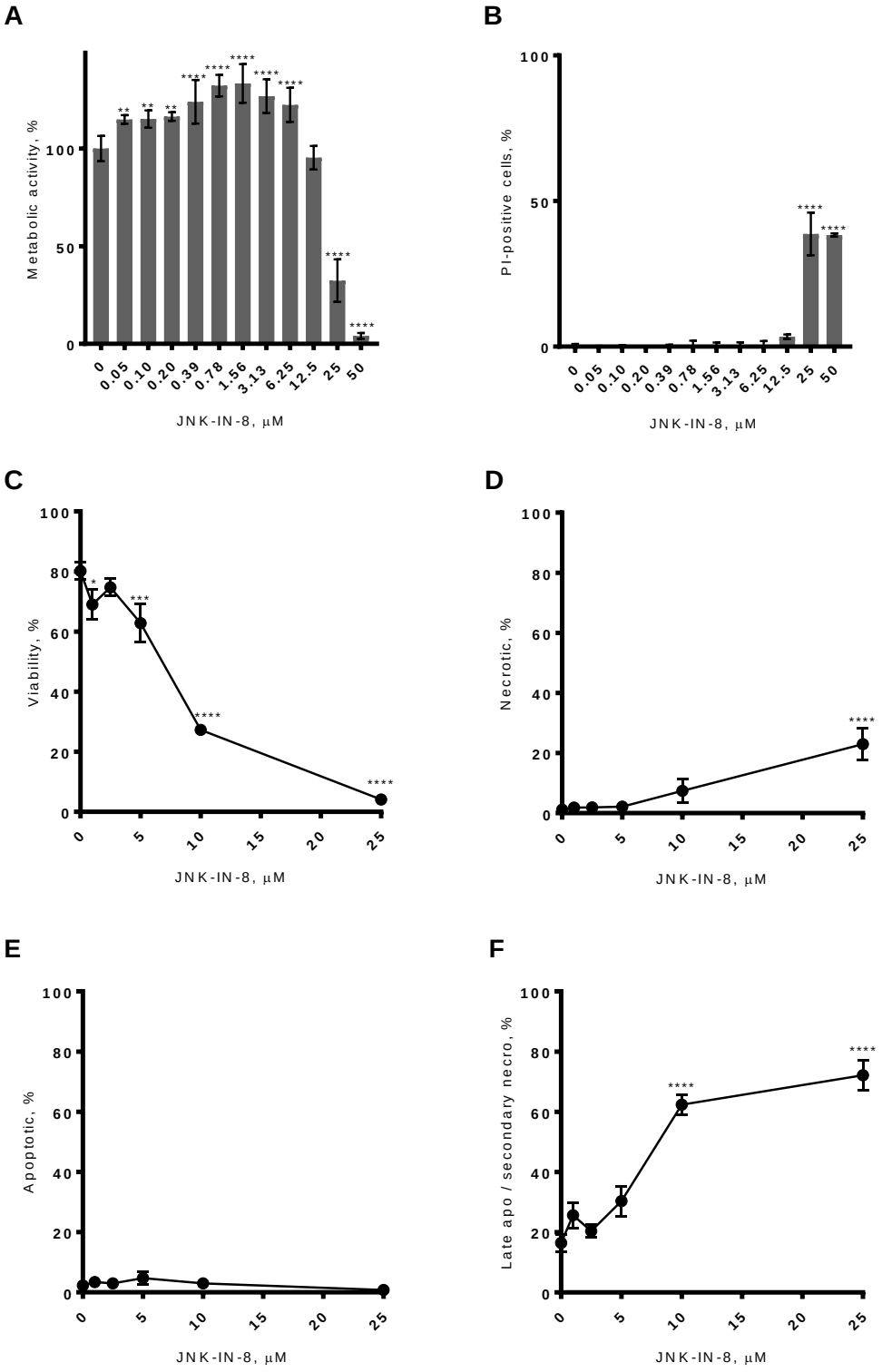

Figure 4.3: Effect of JNK-IN-8 on U937 cell death
U937 cells were treated with varying concentrations of JNK-IN-8 and then incubated at 37°C for 24 h before an (A) MTS assay, (B) PI assay or (C-F) BD Pharmingen™ FITC Annexin V Apoptosis Detection Kit I were performed. Data presented as mean ± SD; n=3. Statistical differences shown to untreated cells, calculated using one-way ANOVA with Dunnett's *post hoc* test: * (P<0.05), *** (P<0.001), **** (P<0.0001).

4.3.1.4 Effect of JNK-IN-8 on MAPK activation

To confirm the effect of JNK-IN-8 on JNK signalling, the effect of 0 to 10 µM JNK-IN-8 on JNK phosphorylation was measured using flow cytometry and Western blotting (Figures 4.4A-C). Flow cytometry results showed the percentages of cells expressing phosphorylated JNK were unaffected by JNK-IN-8 treatment (Figure 4.4A). In the presence of JNK-IN-8, the cells expressing phosphorylated JNK did however express phosphorylated JNK at significantly ($P<0.001$) lower levels (Figure 4.4.B). Western blotting indicated JNK-IN-8 had no effect on JNK phosphorylation (Figure 4.4C).

The effect of JNK-IN-8 on JNK signalling was further investigated by studying the effects of JNK-IN-8 on c-Jun phosphorylation (Figures 4.4D-F). 1 µM JNK-IN-8 did not result in a significant change in the percentage of cells expressing phosphorylated c-Jun (Figure 4.4E) but did cause a significant ($P<0.0001$) decrease in phospho c-Jun MFI (Figure 4.4.F). For 2.5 µM and higher of JNK-IN-8, a dose-dependent inhibition of c-Jun phosphorylation was observed (Figures 4.4E & 4.4F). Western blotting also indicated that c-Jun phosphorylation was inhibited by 1 µM or higher JNK-IN-8 (Figure 4.4D). Taken together, these results suggest that JNK-IN-8, at concentrations as low as 1 µM, inhibit JNK signalling as evidenced by the inhibition of c-Jun phosphorylation, despite limited effect on JNK phosphorylation *per se*.

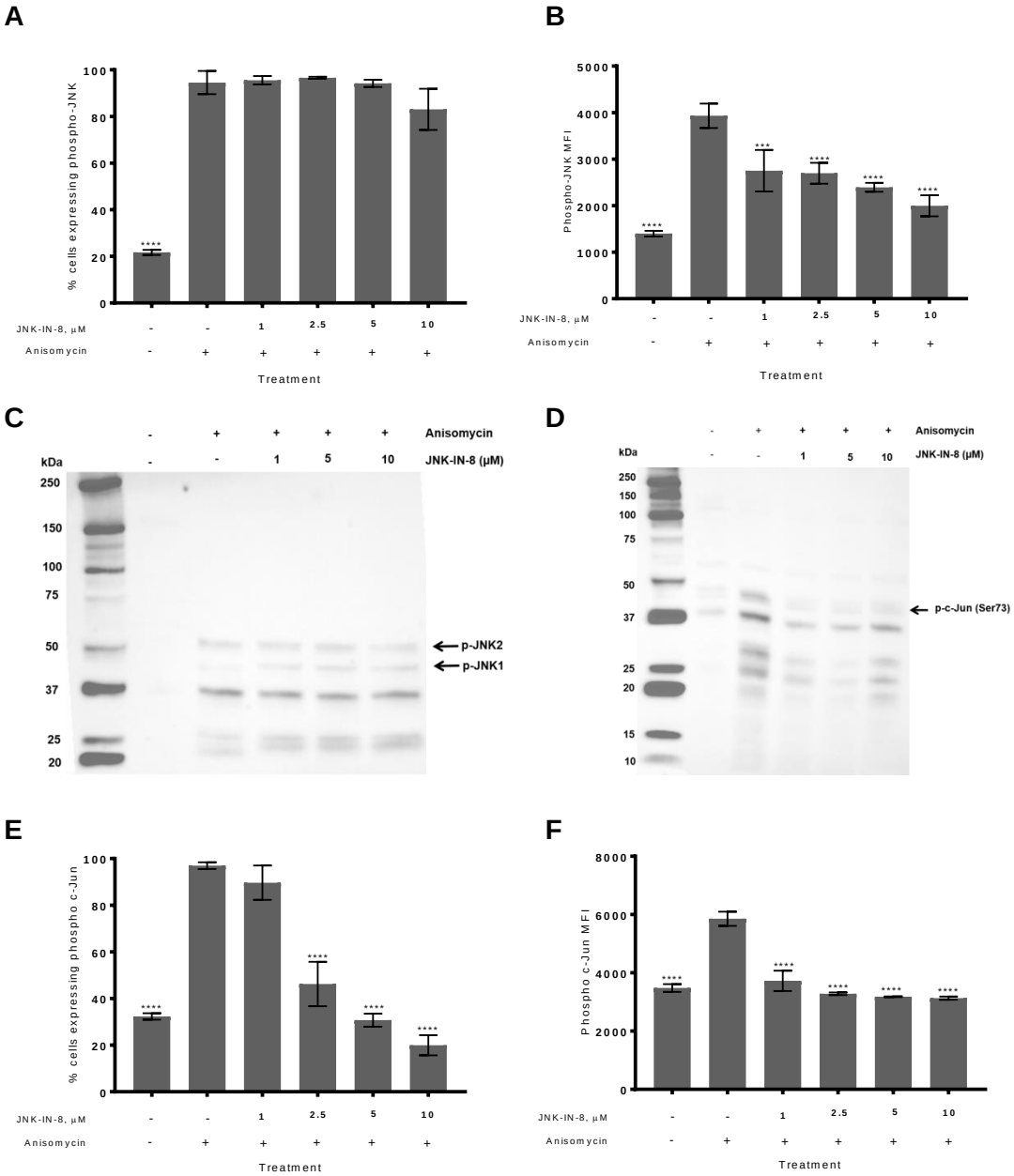

Figure 4.4: Effect of JNK-IN-8 and anisomycin on JNK and c-Jun phosphorylation

U937 cells were pre-treated with various concentrations of JNK-IN-8 at 37°C for 1 h and then incubated with 1 µM anisomycin at 37°C for 30 min. Cells were then prepared for (A,B,E,F) flow cytometry or (C,D) Western blotting. (A,B,E,F) Cells were fixed with 4% paraformaldehyde, permeabilised with 90% ice-cold methanol and stained with (A,B) phospho-SAPK/JNK (Thr183/Tyr185) Alexa Fluor 647-conjugated antibody or (E,F) phospho-c-Jun (Ser73) Alexa Fluor 488-conjugated antibody. (A,E) show the percentage of cells expressing phospho-JNK/c-Jun and (B,F) shows phospho-JNK/c-Jun mean fluorescence intensity (MFI). Data presented as mean ± SD, n=3. Statistical differences shown to anisomycin-only treated cells, calculated using one-way ANOVA with Dunnett's *post hoc* test: *** (P<0.001), **** (P<0.0001). (C,D) Total protein was extracted and separated with SDS-PAGE electrophoresis using 4-15% precast gels. Proteins were electro-transferred to nitrocellulose membrane and (C) phospho-JNK was detected by Western blotting using phospho-JNK/SAPK (Thr183/Tyr185) and (D) phospho-c-Jun was detected using phospho c-Jun (Ser73), and anti-rabbit peroxidase.

4.3.2 Effect of JNK inhibition and UV light

4.3.2.1 Effect of SP600125 and UV light on U937 cell death

To study the role of JNK signalling in UV-induced cell death, U937 cells were treated with a range of UV treatment times in the presence and absence of 10 µM SP600125, and the effect on metabolic activity was assessed. Metabolic activities were significantly ($P<0.0001$) lower for cells pre-treated with 10 µM SP600125 for cells treated with 15 to 50 s UV compared to cells not pre-treated with SP600125 (Figure 4.5A). Results of the PI assay showed that for cells treated with 20 to 40s UV, the percentage of PI-positive cells was significantly ($P<0.0001$) higher for cells treated with SP600125 (Figure 4.5B). The differences observed in the percentage of PI-positive cells (Figure 4.5B) were however less than the differences in metabolic activities (Figure 4.5A) suggesting cell death occurred by a mechanism other than necrosis.

Flow cytometry confirmed that inhibition of JNK signalling, using SP600125, increased the susceptibility of U937 cells to UV-induced reductions in cell viability (Figure 4.5C). The observed differences in cell viabilities were due to differences in the percentages of late apoptotic/ secondary necrotic cells (Figure 4.5E)

Fluorescence microscopy images are consistent with the results of the plate based assays and flow cytometry. In the absence of UV light, U937 cells appeared healthy and did not stain with PI (Figure 4.5F). The majority of cells treated with 6 s UV and not pre-treated with SP600125 appeared apoptotic and PI staining showed these cells had fragmented nuclei. However, a small number of cells appeared viable. For cells treated with 10 µM SP600125, prior to UV light treatment, all cells appeared apoptotic and a large number stained with PI and showed fragmented nuclei. Collectively, these results show SP600125 increases the susceptibility of U937 cells to cell death induced by UV light.

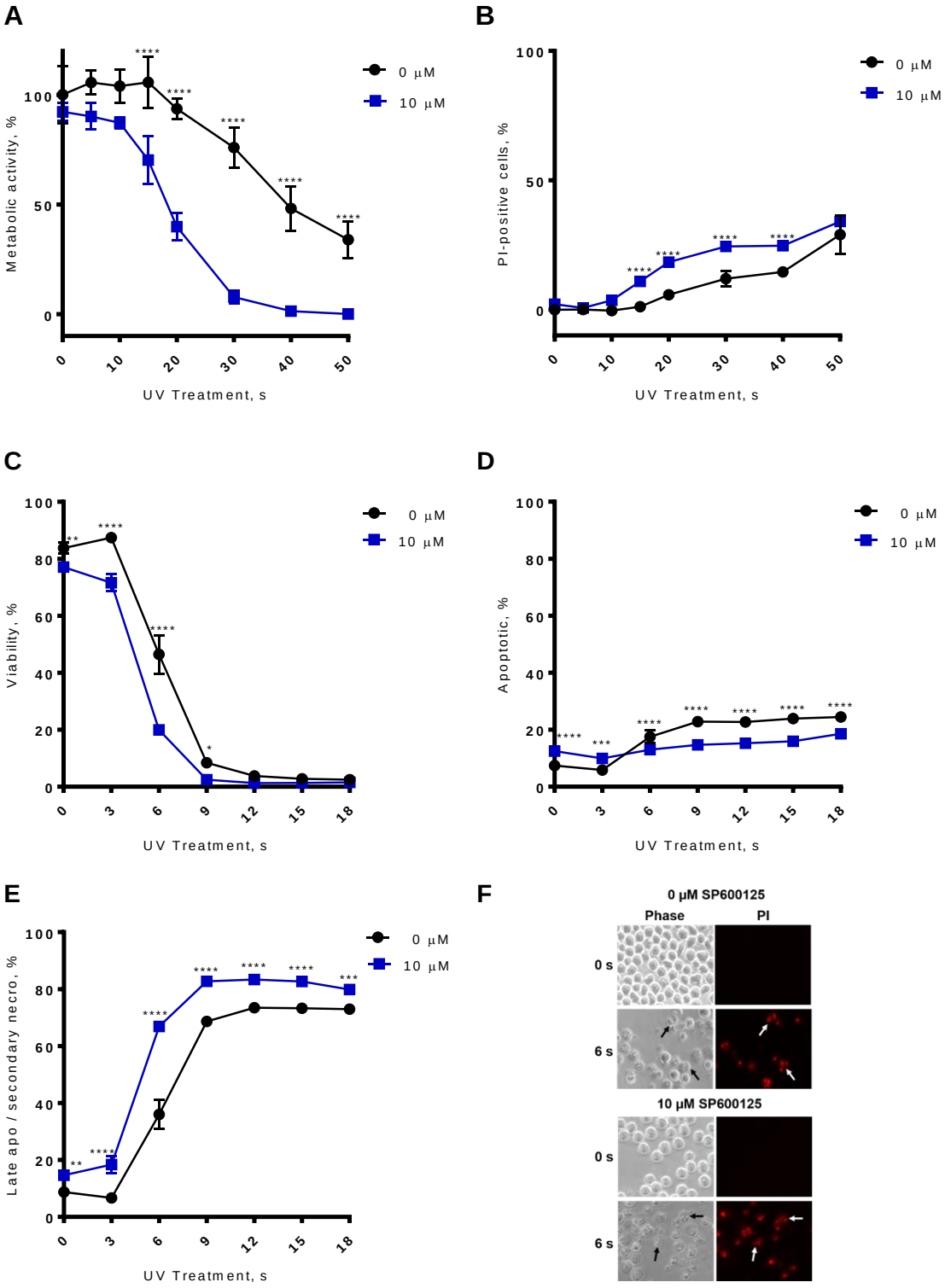

Figure 4.5: Effect of SP600125 and UV light treatment on U937 cell death
U937 cells were pre-treated with 0 or 10 µM SP600125 at 37°C for 1 h to inhibit JNK signalling and then treated with various durations of UV light. After 24 h, (A) MTS assay, (B) PI assay, (C-E) BD Pharmingen™ FITC Annexin V Apoptosis Detection Kit I or (F) microscopy was performed. (A-E) Data presented as mean ± SD, n=3. Statistical differences shown between SP600125 and no treatment at UV time points, calculated using two-way ANOVA with Sidak's *post hoc* test: * (P<0.05), ** (P<0.01), *** (P<0.001), **** (P<0.0001). (F) Cells were stained with PI staining solution and visualised using EVOS™FL AutoCell Imaging System at 40 X magnification. Black and white arrows indicate late apoptotic cells. Images shown are representative of 3 individual images collected.

4.3.2.2 Effect of JNK-IN-8 and UV light on U937 cell death

There were no significant differences in metabolic activities between cells treated with and without JNK-IN-8, irrespective of UV dose (Figure 4.6A and Table 4.1). Hence, for each duration of UV light treatment, there were no significant differences in the percentages of necrotic cells, between cells treated with and without JNK-IN-8 (Figure 4.6B and Table 4.1). As no differences were observed, the effect of JNK-IN-8 and UV light on U937 cell death was not investigated further.

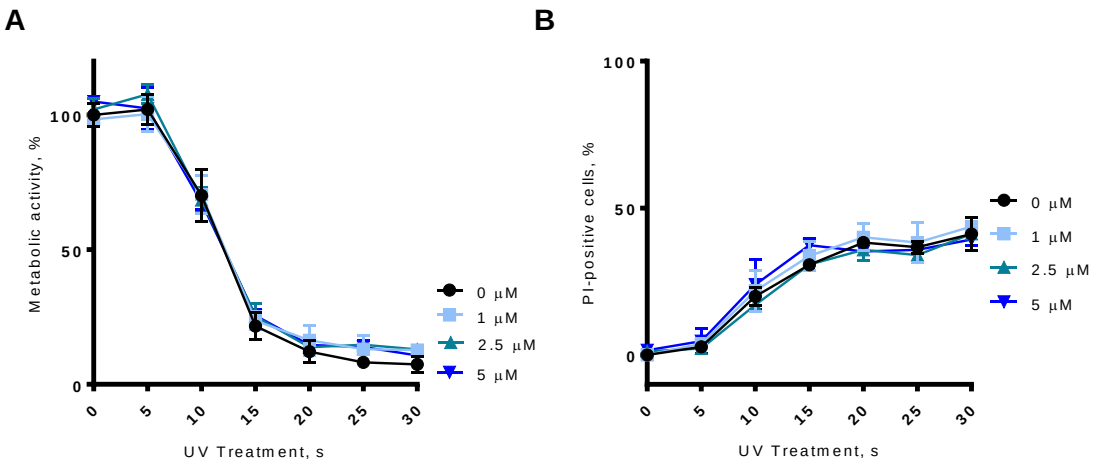

Figure 4.6: Effect of JNK-IN-8 and UV light treatment on U937 metabolic activity and necrosis

U937 cells were pre-treated with 0, 1, 2.5 or 5 µM JNK-IN-8 at 37°C for 1 h to inhibit JNK signalling and then treated with various durations of UV light. After 24 h incubation at 37°C, an (A) MTS assay and (B) PI assay were performed. Data presented as mean ± SD; n=3.

Table 4.1: Statistical analysis to show the effects of JNK-IN-8 and UV light treatment on U937 metabolic activity and necrosis

Data presented as mean ± SD; n=3. Statistical differences shown between JNK-IN-8 and no inhibitor at UV time points, calculated using two-way ANOVA with Tukey's *post hoc* test.

MTS assay			
UV Treatment, s	**JNK-IN-8 concentration**		
	0 µM vs 1 µM	**0 µM vs 2.5 µM**	**0 µM vs 5 µM**
0	ns	ns	ns
5	ns	ns	ns
10	ns	ns	ns
15	ns	ns	ns
20	ns	ns	ns
25	ns	ns	ns
30	ns	ns	ns
PI assay			
UV Treatment, s	**JNK-IN-8 concentration**		
	0 µM vs 1 µM	**0 µM vs 2.5 µM**	**0 µM vs 5 µM**
0	ns	ns	ns
5	ns	ns	ns
10	ns	ns	ns
15	ns	ns	ns
20	ns	ns	ns
25	ns	ns	ns
30	ns	ns	ns

4.3.3 Effect of JNK inhibition and chemotherapy

4.3.3.1 Effect of SP600125 and doxorubicin on U937 cell death

To investigate the role of JNK signalling in doxorubicin-induced cell death, U937 cells were treated with SP600125 prior to doxorubicin treatment, and the effect on metabolic activity was assessed. For cells treated with 0 to 3.13 µM doxorubicin, metabolic activities were significantly ($P<0.05$) lower for cells pre-treated with SP600125 compared to cells not treated with SP600125 (Figure 4.7). As the concentration of doxorubicin increased from 0 to 3.13 µM, the differences in metabolic activity between cells pre-treated with and without SP600125 decreased. For 6.25 and 12.5 µM doxorubicin, metabolic activities were significantly ($P<0.001$) higher for cells pre-treated with SP600125, compared to cells not pre-treated with SP600125. These results suggest that inhibition of JNK signalling provides protection against doxorubicin-induced cell death at least at concentrations of 12.5 µM doxorubicin.

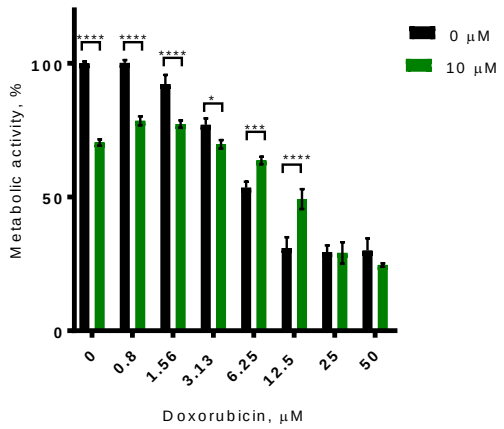

Figure 4.7: Effect of SP600125 and doxorubicin on U937 metabolic activity
U937 cells were pre-treated with 0 or 10 µM SP600125 at 37°C for 1 h to inhibit JNK signalling and then treated with various concentrations of doxorubicin. Cells were then incubated at 37°C for 24 h before an MTS assay was performed. Data presented as mean ± SD; n=3. Statistical differences shown between SP600125 and no inhibitor at doxorubicin concentration points, calculated using two-way ANOVA with Sidak's *post hoc* test: * ($P<0.05$), *** ($P<0.001$), **** ($P<0.0001$).

4.3.3.2 Effect of JNK-IN-8 and doxorubicin on U937 cell death

The role of JNK signalling in doxorubicin-induced cell death was investigated further using JNK-IN-8 in combination with doxorubicin. For cells treated with 0 to 6.25 µM doxorubicin, there were no significant differences in metabolic activities between cells treated with JNK-IN-8 and cells not treated with JNK-IN-8 (Figure 4.8A). For cells treated with 12.5 to 50 µM doxorubicin, metabolic activities were significantly ($P<0.0001$) higher for cells pre-treated with JNK-IN-8 compared to cells not pre-treated with JNK-IN-8, when treated with equivalent concentrations of doxorubicin.

As differences in metabolic activities were observed for cells treated with 12.5 to 50 µM doxorubicin (Figure 4.8A), the effect of JNK-IN-8 and treatment with these concentrations of doxorubicin, was investigated further (Figure 4.8B). For doxorubicin treated cells, active caspase levels were lower for cells pre-treated with JNK-IN-8 compared to cells treated with equivalent concentration of doxorubicin, but not pre-treated with JNK-IN-8. Active caspase levels were however only significantly ($P<0.05$) lower for cells treated with 12.5 µM doxorubicin. These results suggest that either the differences observed in metabolic activities (Figure 4.8A) were not due to apoptosis, or due to the low fluorescence readings for the caspase-3/7 assay (Figure 4.8B), it is possible that 9 h was not the correct time point to measure the activation of caspase-3/7 following doxorubicin treatment.

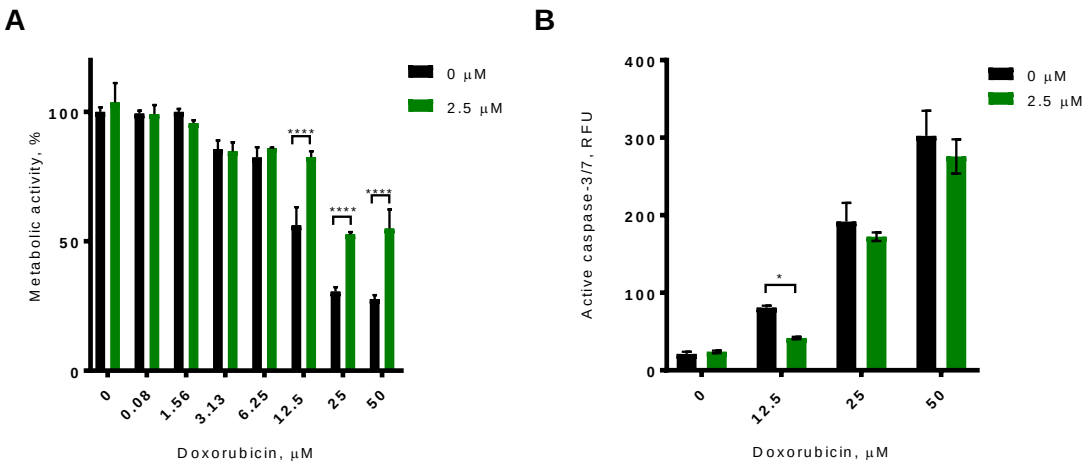

Figure 4.8: Effect of JNK-IN-8 and doxorubicin on U937 cell death
U937 cells were pre-treated with 0 or 2.5 µM JNK-IN-8 at 37°C for 1 h to inhibit JNK signalling and then treated with various concentrations of doxorubicin. Cells were then incubated at 37°C for (A) 24 h before an MTS assay was performed or (B) 9 h before active caspase-3/7 levels were measured using SensoLyte® Homogeneous Rh110 Caspase-3/7 Assay Kit. Data presented as mean ± SD; n=3. Statistical differences shown between JNK-IN-8 and no inhibitor at doxorubicin concentration points, calculated using two-way ANOVA with Sidak's *post hoc* test: * (P<0.05), **** (P<0.0001).

4.3.3.3 Effect of SP600125 and vincristine on U937 cell death

The effect of SP600125 and an alternative chemotherapeutic agent, vincristine on U937 cell death was investigated. For cells treated with 0 to 0.4 nM, metabolic activities were significantly ($P<0.0001$) lower for cells pre-treated with SP600125 compared to cells not treated with SP600125 (Figure 4.9A)
In contrast, for cells treated with 1.56 to 50 nM vincristine, metabolic activities were significantly ($P<0.001$) higher for cells pre-treated with SP600125 compared to cells not treated with SP600125, when treated with equivalent concentrations of vincristine. Results of the PI assay showed that for cells treated with 3.13 to 50 nM vincristine, the percentages of necrotic cells were significantly ($P<0.001$) lower for cells pre-treated with SP600125 compared to cells not treated with SP600125 (Figure 4.9B).

To confirm the effects of SP600125 and vincristine on U937 cell death, flow cytometry was performed to measure annexin V binding and PI staining. Flow cytometry confirmed inhibition of JNK signalling, using SP600125 protected U937 cells from vincristine-induced cell death (Figures 4.9C-E). Flow cytometry confirmed the differences in cell viabilities (Figure 4.9C) were not due to necrosis (data not shown), as suggested by the PI assay (Figure 4.9B) but were due to differences in the percentages of late apoptotic/ secondary necrotic cells (Figure 4.9E).

These results are further supported by the fluorescence microscopy images (Figure 4.9F). For cells treated with 12.5 nM vincristine, and not pre-treated with SP600125, a large number of cells appeared mis-shapen, apoptotic bodies were visible and PI staining showed fragmented nuclei. For cells pre-treated with SP600125 prior to vincristine treatment, a few cells appeared apoptotic but the majority appeared healthy and did not stain with PI. Taken together, these results show that SP600125 protects U937 cells from vincristine-induced cell death.

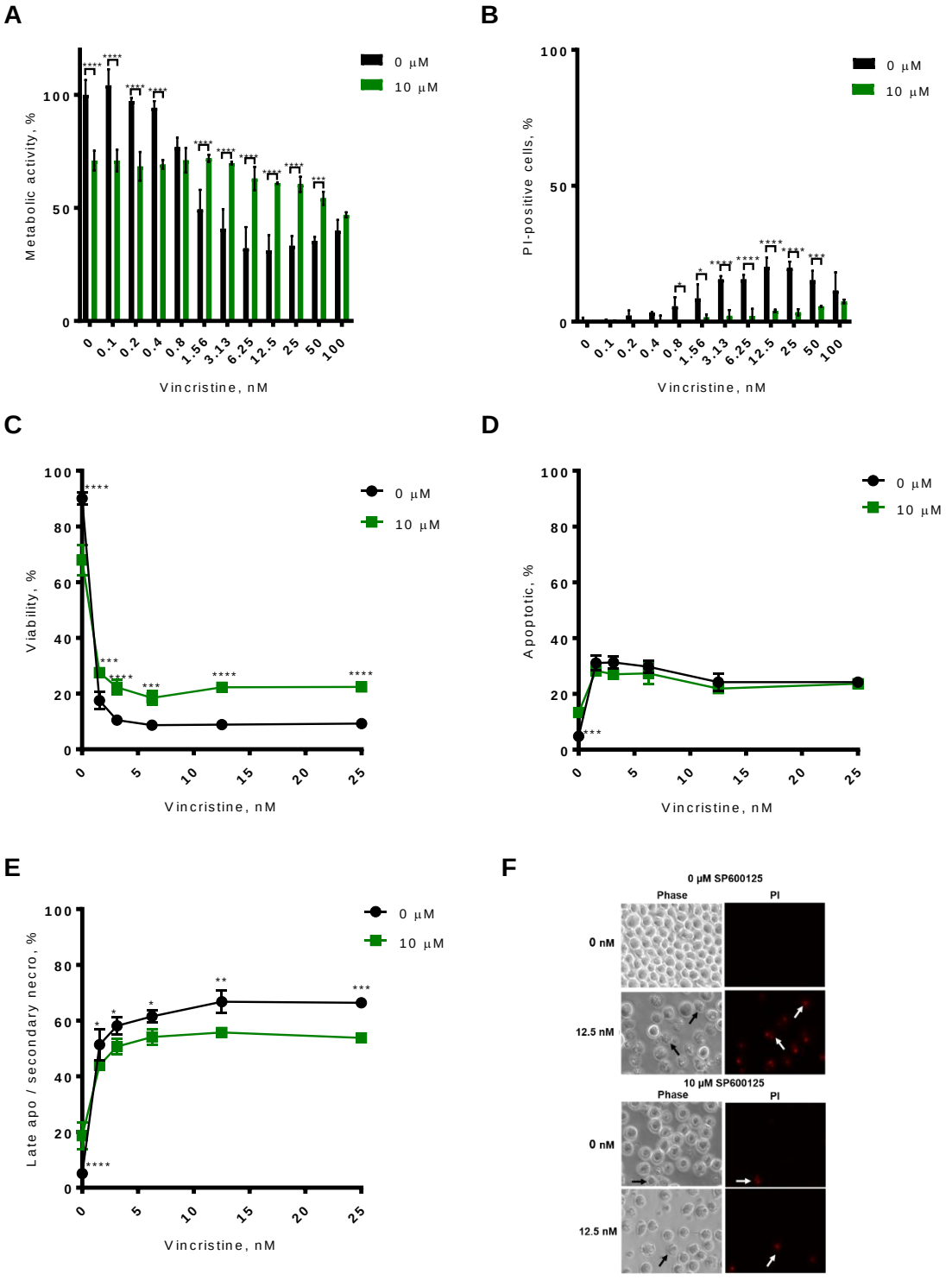

Figure 4.9: Effect of SP600125 and vincristine on U937 cell death

U937 cells were pre-treated with 0 or 10 µM SP600125 at 37°C for 1 h to inhibit JNK signalling and then treated with various concentrations of vincristine. Cells were then incubated at 37°C for 24 h before an A) MTS assay, B) PI assay, (C-E) BD Pharmingen™ FITC Annexin V Apoptosis Detection Kit I or (F) microscopy were performed. (A-E) Data presented as mean ± SD; n=3. Statistical differences shown between SP600125 and no inhibitor at vincristine concentration points, calculated using two-way ANOVA with Sidak's *post hoc* test: * (P<0.05), ** (P<0.01), *** (P<0.001), **** (P<0.0001). (F) Cells were stained with PI staining solution and visualised using EVOS™FL AutoCell Imaging System at 40 X magnification. Black and white arrows indicate late apoptotic cells. Images shown are representative of 3 individual images collected.

4.3.3.4 Effect of JNK-IN-8 and vincristine on U937 cell death

The role of JNK signalling in vincristine-induced cell death was investigated further by pre-treating cells with JNK-IN-8 prior to vincristine and measuring the effects on metabolic activity and necrosis. For cells treated with 0.1 to 0.8 nM vincristine, metabolic activities were significantly ($P<0.05$) lower for cells pre-treated with 2.5 µM JNK-IN-8 compared to cells not treated with JNK-IN-8 (Figure 4.10A). However, a similar difference was measured in the absence of vincristine; the cells appeared to be more susceptible to 2.5 µM JNK-IN-8 than in Figure 4.3. For cells treated with 1.56 to 100 nM vincristine, there were no significant differences in metabolic activities between cells treated with and without JNK-IN-8.

For cells treated with 0 to 0.8 nM vincristine, there were no significant differences in the percentages of necrotic cells between cells treated with and without JNK-IN-8 (Figure 4.10B). For cells treated with 1.56 to 100 nM, the percentages of necrotic cells were significantly ($P<0.05$) lower for cells pre-treated with JNK-IN-8 compared to cells not pre-treated with JNK-IN-8.

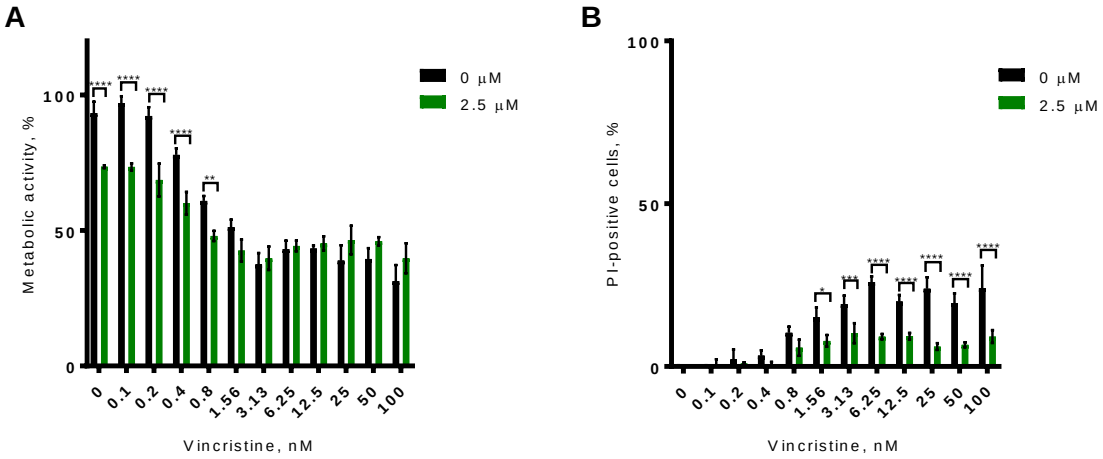

Figure 4.10: Effect of JNK-IN-8 and vincristine on U937 metabolic activity and necrosis

U937 cells were pre-treated with 0 or 2.5 µM JNK-IN-8 at 37°C for 1 h to inhibit JNK signalling. Cells were then treated with various concentrations of vincristine. Cells were then incubated at 37°C for 24 h before an (A) MTS assay or (B) PI assay was performed. Data presented as mean ± SD; n=3. Statistical differences shown between JNK-IN-8 and no inhibitor at vincristine concentration points, calculated using two-way ANOVA with Sidak's *post hoc* test: * ($P<0.05$), ** ($P<0.01$), *** ($P<0.001$), **** ($P<0.0001$).

4.4.4 Effect of JNK inhibition and heat treatment

4.4.4.1 Effect of SP600125 and heat treatment on U937 cell death

To investigate the role of JNK signalling in the response to heat treatment, U937 cells were treated with SP600125 prior to heat treatment and the effect on metabolic activity was assessed. After 24 h, for cells pre-treated with 0 to 3.13 µM SP600125, metabolic activities were significantly ($P<0.01$) lower for heat treated cells compared to non-heat treated cells (Figure 4.11A). Metabolic activities were significantly ($P<0.01$) higher for heat treated cells compared to non-heat treated cells treated with 12.5 and 50 µM SP600125. Although statistically significant, the differences were minimal. Heat treatment also had minimal effects on necrosis after 24 h and this was unaffected by SP600125 (data not shown)

After 24 h, flow cytometry results showed that the percentages of viable cells were lower for heat treated cells compared to non-heat treated cells, for both cells treated with and without SP600125 (Figure 4.11B). The differences observed were due to small increases in the percentages of apoptotic and late apoptotic/secondary necrotic cells for heat treated cells (Figures 4.11C-D).

As heat shock was selected as a stimulus to stress the cells, rather than directly induce cell death, the effect of SP600125 and heat treatment was also studied after a longer time period of 48 h. After 48 h, for cells treated with 0 to 6.25 µM SP600125, there were no significant differences in metabolic activities between heat treated and non-heat treated cells (Figure 4.11E). Metabolic activities were significantly ($P<0.05$) higher for heat treated cells compared to non-heat treated cells for cells pre-treated with 12.5 to 50 µM SP600125 after 48 h. SP600125 and heat treatment had little effect on U937 necrosis after 48 h (data not shown).

After 48 h, flow cytometry results showed there were no significant differences in cell viabilities between heat treated and non-heat treated cells, for all concentrations of SP600125 (Figure 4.11F). For cells treated with 50 µM SP600125, the percentage of apoptotic cells was significantly ($P<0.001$) lower for heat treated cells, compared to non-heat treated cells, but the percentage of late apoptotic/secondary necrotic was significantly ($P<0.001$) higher (Figures 4.11G-H).

To further investigate the effects of SP600125 and heat treatment on U937 cell death, active caspase-3/7 levels were measured (Figure 4.12).For all concentrations of SP600125, active caspase-3/7 levels were increased by around 2-fold ($P<0.0001$) for heat treated cells compared to non-heat treated cells. Active caspase-3/7 levels were also significantly ($P<0.0001$) increased to a similar extent in the absence of SP600125. Collectively, these results suggest inhibition of JNK signalling using SP600125, has minimal effects on U937 cell death following heat treatment.

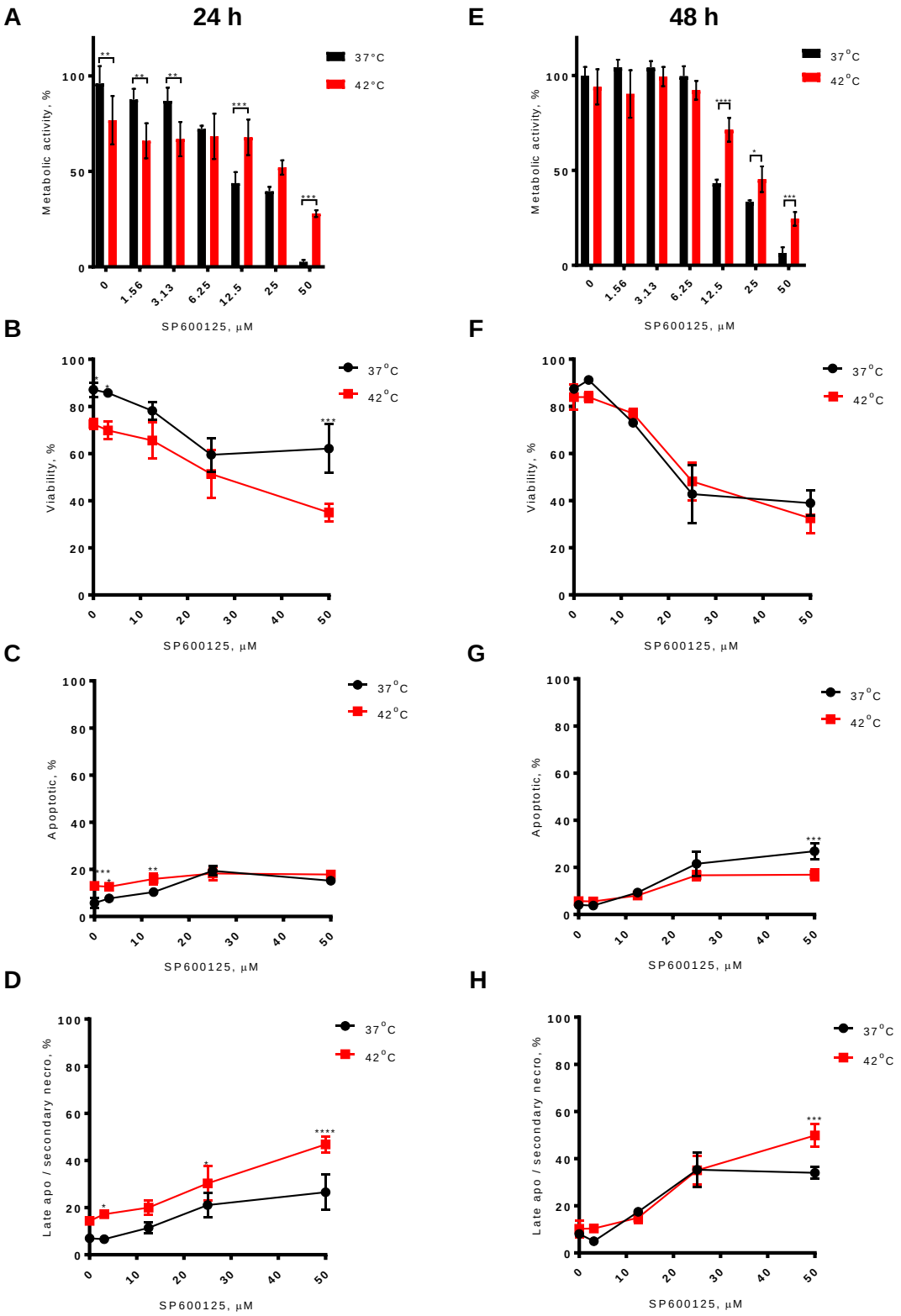

Figure 4.11: Effect of SP600125 & heat treatment on U937 cell death
U937 cells were pre-treated with SP600125 at 37°C for 1 h to inhibit JNK signalling and then incubated at either 37°C or 42°C for 1 h. Cells were then incubated at 37°C for (A-D) 24 h or (E-H) 48h before an (A,E) MTS assay or (B-D,F-H) BD Pharmingen™ FITC Annexin V Apoptosis Detection Kit I were performed. Data presented as mean ± SD; n=3. Statistical differences shown between heat treatment and no treatment at SP600125 concentration points, calculated using two-way ANOVA with Sidak's *post hoc* test: * (P<0.05), ** (P<0.01), *** (P<0.001).

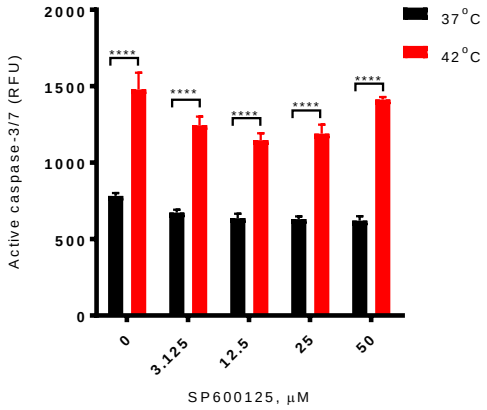

Figure 4.12: Effect of SP600125 and heat treatment on caspase-3/7 activation, U937 cells were pre-treated with SP600125 at 37ºC for 1 h to inhibit JNK signalling and then incubated at either 37°C or 42ºC for 1 h. Cells were then incubated at 37°C for 9 h before active caspase-3/7 levels were measured using SensoLyte® Homogeneous Rh110 Caspase-3/7 Assay Kit. Data presented as mean ± SD, n=3. Statistical differences shown between mean heat treatment and no treatment at SP600125 concentration points, calculated using two-way ANOVA with Sidak's *post hoc* test: **** (P<0.0001).

4.4.3.2 Effect of JNK-IN-8 and heat treatment on U937 cell death

The role of JNK signalling in U937 cell death following heat treatment was investigated further using JNK-IN-8. In the absence of JNK-IN-8, there was no significant difference in metabolic activity between heat treated and non-heat treated cells after 24 h (Figure 4.13A). Metabolic activities were significantly ($P<0.0001$) lower for heat treated cells compared to non-heat treated cells for cells pre-treated with 5 to 25 µM JNK-IN-8. To determine if the observed differences in metabolic activities were due to necrosis, a PI assay was performed (Figure 4.13B). After 24 h the percentages of necrotic cells were significantly ($P<0.0001$) higher for heat treated cells compared to non-heat treated cells when pre-treated with 10 to 25 µM JNK-IN-8.

After 24 h, flow cytometry results showed that cell viabilities were significantly ($P<0.0001$) lower for heat treated cells compared to non-heat treated cells, for both cells treated with and without JNK-IN-8 (Figure 4.13C). The observed differences in cell viabilities (Figure 4.13C) were due to differences in the percentage of late apoptotic/secondary necrotic cells (Figure 4.14D). These differences in the percentages of late apoptotic/secondary necrotic cells accounts for the differences in percentages of PI-positive cells, measured by the PI assay (Figure 4.13B).

After 48 h, for 0 and 5 µM JNK-IN-8, the metabolic activity of heat treated cells was significantly ($P<0.01$) higher compared to non-heat treated cells (Figure 4.13E). However these differences were minimal, less than a 10% difference was measured. Metabolic activities were significantly ($P<0.0001$) lower for heat treated cells compared to non-heat treated cells for cells pre-treated with 10 to 25 µM JNK-IN-8. JNK-IN-8 and heat treatment had little effect on U937 necrosis after 48 h (data not shown).

Flow cytometry results confirmed that after 48 h cell viabilities were significantly ($P<0.0001$) lower for heat treated cells compared to non-heat treated cells, for cells treated with JNK-IN-8 (Figure 4.13F).The percentages of apoptotic and late apoptotic/secondary necrotic cells were significantly ($P<0.05$) higher for heat treated cells compared to non-heat treated cells pre-treated with JNK-IN-8. Collectively, these results indicate JNK-IN-8 makes U937 cells more susceptible to heat-induced cell death after 24 and 48 h.

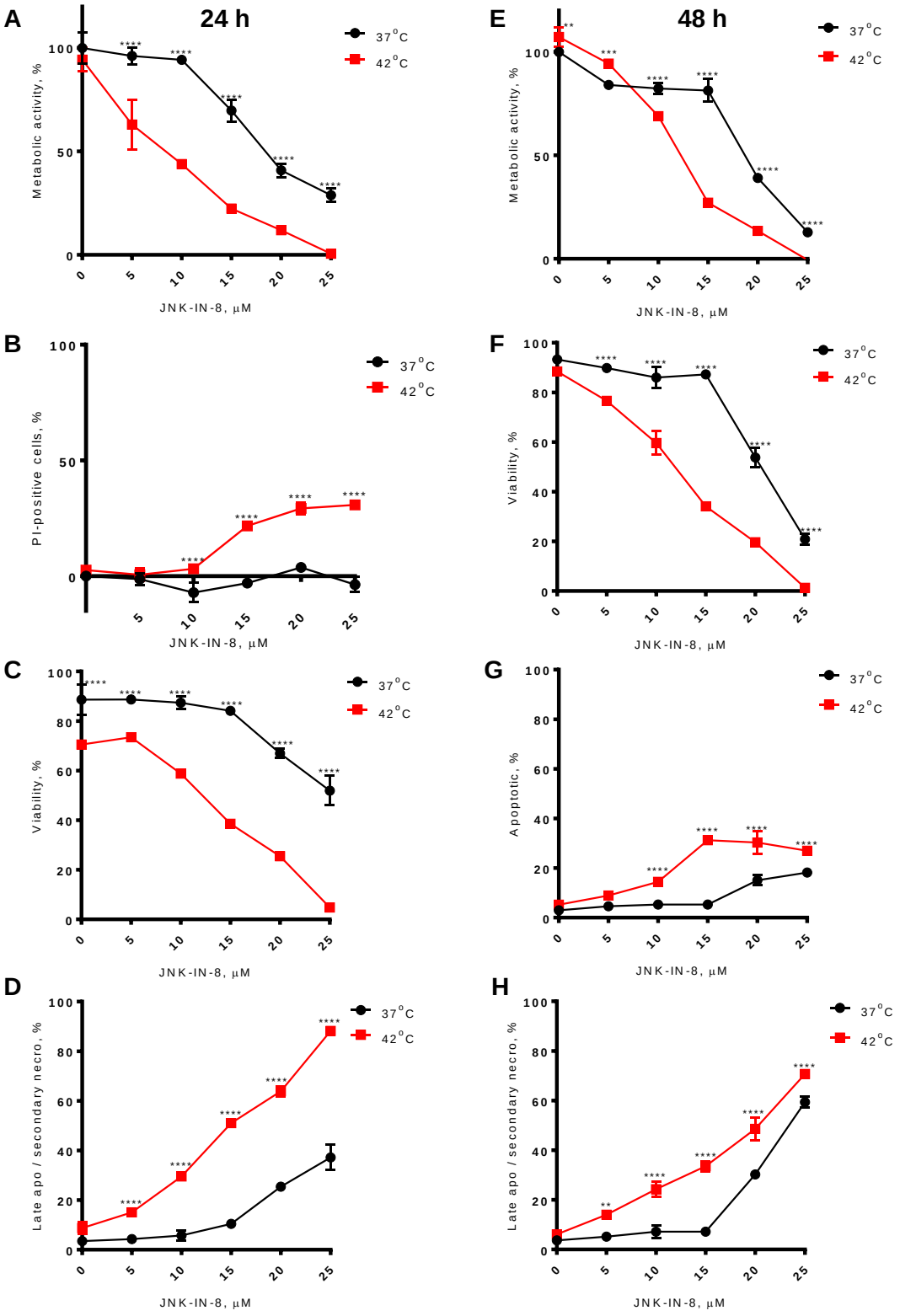

Figure 4.13: Effect of JNK-IN-8 and heat treatment on U937 cell death
U937 cells were pre-treated with JNK-IN-8 at 37°C for 1 h to inhibit JNK signalling and then incubated at either 37°C or 42°C for 1 h. Cells were then incubated at 37°C for (A-D) 24 h or (E-H) 48h before an (A,E) MTS assay, (B) PI assay or (C-D,F-H) BD Pharmingen™ FITC Annexin V Apoptosis Detection Kit I were performed. Data presented as mean ± SD; n=3. Statistical differences shown between heat treatment and no treatment at JNK-IN-8 concentration points, calculated using two-way ANOVA with Sidak's *post hoc* test: * (P<0.05), ** (P<0.01), *** (P<0.001).

To confirm the effects of JNK-IN-8 and heat treatment directly on apoptosis, active caspase-3/7 levels were measured following JNK-IN-8 and heat treatment. First of all it was necessary to establish an appropriate time to measure active caspase-3/7 levels. The time dependent activation of caspase-3/7 was measured following treatment with 15 µM JNK-IN-8, as results presented in figure 4.13 provided a strong indication that this combination of treatments induced apoptosis. Active caspase-3/7 levels were around 5-fold higher ($P<0.0001$) for heat treated cells compared to non-heat treated cells after 0 h (Figure 4.14A). After 3 h, active caspase-3/7 levels were around 13-fold higher ($P<0.0001$) for heat treated cells and these levels were sustained up to 12 h. After 24 h, active caspase-3/7 levels were still significantly ($P<0.0001$) higher for heat treated cells compared to non-heat treated cells but only by around 3-fold. The levels of active caspase-3/7 prior to JNK-IN-8 and heat treatment were however not measured. A further experiment was therefore performed to measure the levels prior to treatment, and up to 9 h after treatment. There was no significant differences in active caspase-3/7 levels between heat treated cells and non-heat treated cells, prior to JNK-IN-8 and heat treatment (Figure 4.14B). After 9 h after heat treatment, active caspase-3/7 levels were around 10-fold higher for heat treated cells compared to non-heat treated cells which is consistent with the previous results (Figure 4.14A). However, the results in Figure 4.15B suggest that for heat treated cells, active caspase-3/7 levels gradually increased in a time-dependent manner between 0 and 9 h.

Active caspase-3/7 levels were therefore measured 9 h after JNK-IN-8 and heat treatment. There was no significant difference in active caspase-3/7 levels between heat treated and non-heat treated cells in the absence of JNK-IN-8 treatment (Figure 4.15). For cells pre-treated with JNK-IN-8, active caspase-3/7 levels were significantly ($P<0.0001$) higher for heat treated cells compared to non-heat treated cells. These results confirm that JNK inhibition, using JNK-IN-8 makes U937 cells susceptible to heat-induced cell death, in particular, susceptible to apoptosis.

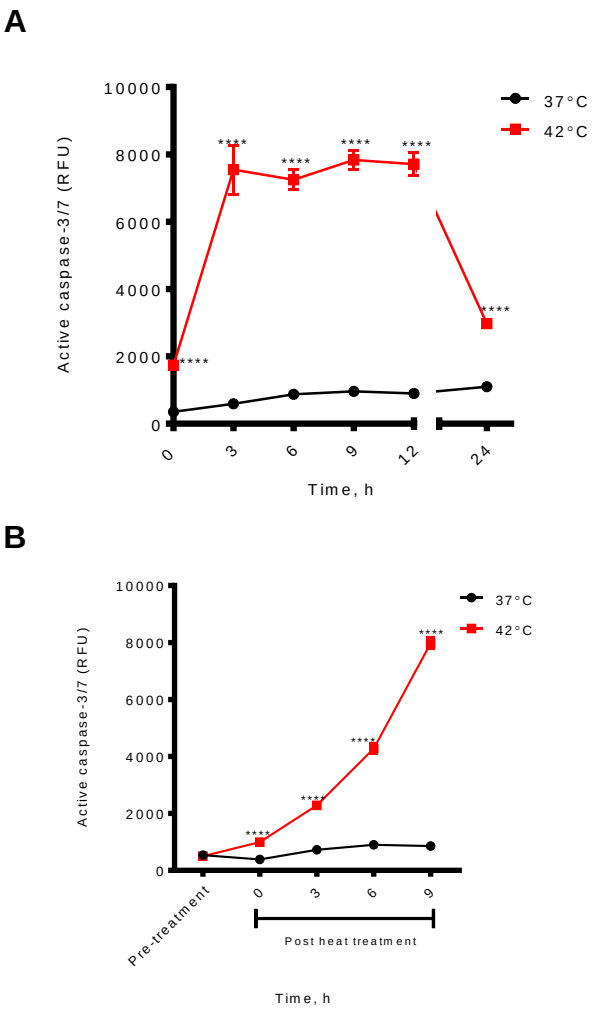

Figure 4.14: Time-dependent activation of caspase-3/7 activation following JNK-IN-8 and heat treatment

U937 cells were pre-treated with 15 µM JNK-IN-8 at 37ºC for 1 h to inhibit JNK signalling and then incubated at either 37°C or 42ºC for 1 h. Cells were then incubated at 37°C and active caspase-3/7 levels were measured over (A) 24 h or (B) 9h using SensoLyte® Homogeneous Rh110 Caspase-3/7 Assay Kit. Data presented as mean ± SD, n=3. Statistical differences shown between heat treatment and no treatment at time points, calculated using two-way ANOVA with Sidak's *post hoc* test: **** (P<0.0001).

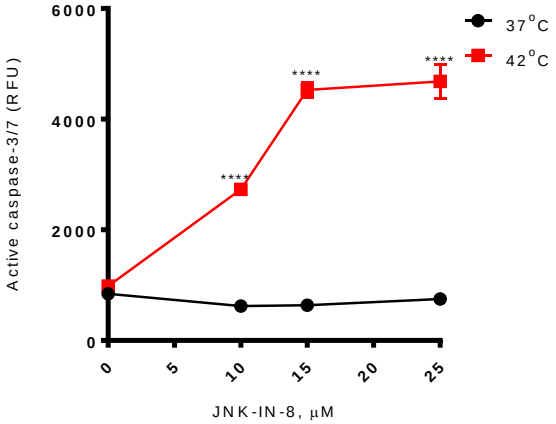

Figure 4.15: Effect of JNK-IN-8 and heat treatment on caspase-3/7 activation
U937 cells were pre-treated with JNK-IN-8 at 37ºC for 1 h to inhibit JNK signalling and then incubated at either 37ºC or 42ºC for 1 h. Cells were then incubated at 37°C for 9 h before active caspase-3/7 levels were measured using SensoLyte® Homogeneous Rh110 Caspase-3/7 Assay Kit. Data presented as mean ± SD, n=3. Statistical differences shown between heat treatment and no treatment at JNK-IN-8 concentration points, calculated using two-way ANOVA with Sidak's *post hoc* test: **** (P<0.0001).

4.4 Discussion

The overall aim of this chapter was to gain a greater understanding of the role of JNK signalling in leukemic cell death by studying the effects of JNK inhibition and cell stressors on cell death in the model U937 cell line.

4.4.1 Inhibition of JNK signalling

The JNK inhibitors, SP600125 and JNK-IN-8, were used to target JNK signalling in this study (Section 4.3.1). SP600125 is an ATP-competitive inhibitor (Bennett et al., 2001) whereas JNK-IN-8 is a covalent, irreversible inhibitor which binds to JNK (Zhang et al., 2012); both inhibitors prevent the phosphorylation of c-Jun, a downstream target of JNK. A large number of studies have published data using SP600125 but its use is restricted due to poor specificity. Due to the more recent discovery of JNK-IN-8, a limited number of studies have utilised JNK-IN-8. The aim here was to confirm the inhibition of JNK signalling in U937 cells using both SP600125 and JNK-IN-8, and to establish effective concentrations to use in combination with cell stressors.

4.4.1.1 SP600125

Results showed that although concentrations of SP00125 up to 25 µM do cause some cell death in U937 cells, the effect is minimal (Section 4.3.1.1). The differences observed in metabolic activities could be due to the ability of SP600125 to affect cellular processes other than apoptosis, such as proliferation. This theory is supported by previous evidence showing SP600125 inhibits proliferation of U937 cells, by causing cells to arrest at G2/M stage of the cell cycle, and inducing endoreduplication (Moon et al., 2009). This is consistent with the ability of SP600125 to inhibit cell growth in other cancer cells (Hideshima et al., 2003; Jacobs-Helber & Sawyer, 2004; Mingo-Sion, Marietta, Koller, Wolf, & Van Den Berg, 2004).

The effect of SP600125 on the phosphorylation of JNK was measured using both flow cytometry and Western blotting (Section 4.3.1.2). JNK phosphorylation was not inhibited by SP600125, as measured by flow cytometry but Western blotting showed JNK phosphorylation was inhibited by 10 and 25 µM SP600125. Therefore, the measurement of JNK

phosphorylation following SP600125 treatment, using two alternative techniques, produced inconsistent results.

As SP600125 is an ATP-competitive JNK inhibitor, it was expected that JNK phosphorylation would be inhibited by SP600125. Bennett et al. have reported that SP600125 inhibits JNK phosphorylation in Jurkat T lymphocyte cells, but the evidence to support this conclusion is not convincing (Bennett et al., 2001). Inhibition of JNK phosphorylation by SP600125 however has previously been reported in U937 cells (Budhraja et al., 2012) and other cancer cells lines such as BKS-2 B cell lymphoma cells (Gururajan et al., 2005) and Hep G2 hepatocellular carcinoma cells (Kong et al., 2014). As in these studies, JNK phosphorylation is typically measured by Western blotting rather than flow cytometry. The observed differences are unlikely to be due to the antibodies used as the antibodies used in the flow cytometry and Western blotting experiments in this chapter, both measured the dual phosphorylation of JNK at residues Thr183 and Tyr185.

Another possible explanation for the observed differences between the two techniques could be attributed to cell numbers. Flow cytometry analyses individual cells whereas Western blotting involves the extraction of proteins from a sample of cells. As SP600125 inhibits cell proliferation (Moon et al., 2009), it is possible the decreased levels of phosphorylated JNK following SP600125 treatment could be due to a lower number of cells compared to untreated cells. However the short time course of this experiment was unlikely to have a significant effect on proliferation and subsequent cell numbers, to cause such effects. The loading of equal concentrations of protein onto the gels should also have accounted for differences in cell numbers; measurement of total JNK would have provided confirmation of equal loading.

Western blotting measures the levels of phosphorylated JNK expressed by a population of cells, including both viable and non-viable cells. The flow cytometry data presented however only measured the percentage of viable cells expressing phosphorylated JNK and non-viable cells were excluded from analysis. Analysis of the percentage of total cells (data not shown) expressing phosphorylated JNK, showed inhibition of JNK phosphorylation in SP600125-treated cells, consistent with the Western

blotting data. These results therefore suggest JNK phosphorylation is not inhibited by SP600125 in viable cells.

Further confirmation was therefore required regarding the ability of SP600125 to inhibit JNK signalling. SP600125 prevents the ability of JNK to phosphorylate downstream targets and c-Jun has been identified as an affected target; SP600125 inhibits c-Jun phosphorylation in a dose-dependent manner (Bennett et al., 2001). The phosphorylation of c-Jun, at Ser73, was measured following SP600125 and anisomycin treatment. As expected, flow cytometry showed c-Jun phosphorylation was inhibited by all concentrations of SP600125 tested. These results provide clarification that SP600125 can inhibit JNK signalling in U937 cells by preventing the phosphorylation of c-Jun. Due to the minimal effect on cell viability and the ability to inhibit JNK signalling, 10 µM SP600125 was selected for use in future experiments.

4.4.1.2 JNK-IN-8

There are a limited number of published studies using JNK-IN-8 in cancer cells lines and no reports of its use specifically in U937 cells. High concentrations of JNK-IN-8 (greater than 5 µM) caused significant cell death in U937 cells and were therefore deemed unsuitable concentrations for this study. Low concentrations of JNK-IN-8 (less than 6.25 µM) did cause an increase in metabolic activity but this is unlikely to be due to the ability of JNK-IN-8 to enhance cell proliferation as c-Jun phosphorylation is critical for cell proliferation (Shaulian & Karin, 2001). A small decrease in cell viability and increase in late apoptosis/ secondary necrosis was observed following treatment with low concentrations of JNK-IN-8, which provides a possible explanation for the increase in metabolic activity as apoptosis is a metabolically active process (Elmore, 2007).

During the development of the inhibitor, it was shown that JNK phosphorylation was not inhibited by JNK-IN-8 in human embryonic kidney 293 (HEK293) cell line (Zhang et al., 2012). However a literature search shows this is the only cell line tested. Flow cytometry results showed the percentage of cells expressing phosphorylated JNK was decreased by JNK-IN-8 whilst Western blotting however showed JNK-IN-8 had no effect on JNK phosphorylation. Due to the mechanism of action of JNK-IN-8, it was not

expected that JNK phosphorylation would be affected by JNK-IN-8 treatment. However JNK-IN-8 could affect the phosphorylation of JNK indirectly due to signalling feedback mechanisms within the cells.

The effect of JNK-IN-8 on the phosphorylation of c-Jun was measured. Flow cytometry results showed that c-Jun phosphorylation was inhibited by JNK-IN-8. Western blotting confirmed c-Jun phosphorylation was inhibited by JNK-IN-8, even at 1 µM. Inhibition of c-Jun phosphorylation by 1 µM or higher of JNK-IN-8 in U937 cells is in agreement with previous reports of c-Jun inhibition using similar concentrations of JNK-IN-8 in other cancer cells lines (Cerezo-Guisado et al., 2015; Zhang et al., 2012). Although these previous studies measured the phosphorylation of c-Jun at Ser63, whereas the antibody used in this study recognised c-Jun phosphorylated at Ser73, the results are consistent.

Concentrations of JNK-IN-8 of less than 5 µM have typically been used in the studies utilising JNK-IN-8 in other cancer cell lines (Cerezo-Guisado et al., 2015; Fallahi-Sichani et al., 2015; Hsiao et al., 2014; Zhou et al., 2016). As the results of this study showed 1 and 2.5 µM JNK-IN-8 inhibited c-Jun phosphorylation, without having a large effect on cell viability, these concentrations of JNK-IN-8 were selected to use in combination with cell stressors.

4.4.2 JNK inhibition and UV

Inhibition of JNK signalling, using SP600125 made the U937 cells more susceptible to cell death induced by UV (Section 4.3.2.1), suggesting a protective role of JNK signalling against UV-induced apoptosis in U937 cells. Pre-treatment with the alternative JNK inhibitor, JNK-IN-8, however had no effect on cell death induced by UV (Section 4.3.2.2). These results were surprising, given that such striking differences were observed using SP600125 (Section 4.3.2.1) and JNK-IN-8 is more effective at inhibiting JNK signalling than SP600125 (Section 4.3.1). It could be possible JNK signalling provides protection against UV-induced apoptosis via a pathway that involves JNK phosphorylation but not c-Jun phosphorylation. However, as JNK-IN-8 showed ability to inhibit JNK phosphorylation (Section 4.3.1.4), it was expected similar effects would have been observed for JNK-IN-8, as for SP600125. It is

possible the different effects observed using SP600125 and JNK-IN-8 could be related to differences in the specificities of the inhibitors.

Whilst, it was originally believed SP600125 was selective for JNK (Bennett et al., 2001), other studies have since argued SP600125 is not specific for JNK (Bain, Plate, et al., 2007). In fact, SP600125 affects a range of other kinases with similar or greater potency than JNK. It is possible treatment with SP600125 inhibits JNK phosphorylation but causes activation of other signalling pathways involved in apoptosis. In contrast, JNK-IN-8 has been reported to be a highly selective inhibitor (Zhang et al., 2012), although the specificity has not been directly tested in U937 cells. Measuring the effects of JNK inhibition and UV treatment on the activation of JNK and other kinases would provide a greater understanding of the role of JNK signalling in UV-induced apoptosis in U937 cells.

It is well established UV activates the JNK signalling pathway which results in induction of apoptosis. The results presented in this chapter however suggest JNK signalling has a protective role against UV-induced apoptosis in U937 which contradicts previous findings in other cancer cell lines. JNK inhibition prevents UV-induced cell death in MCF-7 and MB-231 breast cancer cells (Mingo-Sion, Marietta, Koller, Wolf, Van, et al., 2004). PARP cleavage, an indicator of cell death, was induced by UV in both MCF-7 and MB-231 cells, and PARP cleavage was inhibited by SP600125. SP600125 was less effective at inhibiting UV-induced cell death in MB-231 cells compared to MCF-7 cells, which highlights the variability between cell lines within a particular disease. The study also demonstrated the involvement of c-Jun in response of MCF-7 cells to UV; c-Jun was activated following UV treatment and was inhibited by SP600125. Inhibition of JNK, using SP600125 also prevented UV-induced apoptosis in ASTC-a-1 human lung adenocarcinoma cells (Li, Liu, Xing, & Chen, 2010). SP600125 delayed the mitochondrial translocation of the pro-apoptotic protein Bax following UV treatment. JNK inhibition was achieved in both of these studies using SP600125, the same inhibitor as used in this study. As the results presented in this chapter conflict the results of these studies, and all studies utilised SP600125, the observations in this chapter are unlikely to be due to the poor specificity of SP600125 as previously discussed. Confirmation of the effects of SP600125 and UV treatment on MAPK

phosphorylation would provide clarification on this matter. It however appears that the role of JNK in UV-induced apoptosis is cell line specific. SP600125 had no effect on UV-induced apoptosis in SH-SY5Y neuroblastoma cells. Despite inhibition of UV-induced phosphorylation of c-Jun by SP600125 (Berglund, Radesäter, Persson, & Budd Haeberlein, 2004), caspase-3 activity and cytochrome c release were unaffected.

As very different effects are observed following treatment with the JNK inhibitors SP600125 and JNK-IN-8, further investigations are required in order to confirm the involvement of JNK signalling in UV-induced cell death in U937 cells. The use of alternative, selective inhibitors or JNK knockouts would provide clarification.

4.4.3 JNK inhibition and chemotherapeutic agents

Chemotherapy can induce apoptosis in cancer cells through the activation of JNK signalling, and the failure to activate JNK has been associated with chemotherapy resistance. The role of JNK signalling in response to chemotherapy appears to be specific to the type of cancer and the chemotherapeutic agent used. The aim here was to gain a greater understanding of the role of JNK signalling in doxorubicin and vincristine-induced apoptosis in U937 cells.

4.4.3.1 Doxorubicin

Inhibition of JNK signalling, using SP600125, appeared to provide a level of protection against the doxorubicin-induced reduction in metabolic activity (Section 4.3.3.1). However, in order to draw valid conclusions from this data, further confirmation of the effect of SP600125 and doxorubicin on apoptosis is required. Protection against doxorubicin-induced reductions in metabolic activities were also observed when JNK signalling was inhibited using JNK-IN-8 (Section 4.3.4.2). Measurement of active-caspase3/7 levels indicated that the differences in metabolic activities were due to differences in apoptosis. Lower levels of active caspase-3/7 were measured for doxorubicin-treated cells pre-treated with JNK-IN-8 compared to cells not pre-treated with JNK-IN-8. However further work is needed to measure the time-dependent activation of caspase-3/7 following doxorubicin treatment.

Collectively, the results of JNK signalling inhibition, using SP600125 or JNK-IN-8, prior to doxorubicin treatment, provide a strong indication that JNK signalling plays an important role in doxorubicin-induced cell death. This study however only assessed the effects of JNK signalling inhibition and doxorubicin treatment on U937 cell viability. Measurement of the activation of JNK signalling components following these treatments would provide a greater insight into the mechanisms and involvement of JNK signalling in doxorubicin-induced apoptosis in U937 cells. The activation of JNK following doxorubicin treatment has previously been reported in U937 cells (Lagadinou et al., 2008).

Lagadinou et al. (20008) also showed that downregulation of JNK1 resulted in lower percentages of apoptotic cells following doxorubicin treatment compared to cells in which JNK signalling had not been inhibited, highlighting the importance of the JNK1 isoform. The results presented in this chapter support the findings of Lagadinou et al. (2008) but also suggest an important role of c-Jun phosphorylation. There is promising evidence that doxorubicin resistance can be reversed by the stimulation of JNK signalling. JNK was not activated in doxorubicin-resistant U937 cells but activation of JNK signalling in these resistant cells, by constitutive activation of MKK4, sensitised the cells to doxorubicin (Lagadinou et al., 2008). *In vivo* results show JNK activation failure in bone marrow cells from AML patients is associated with resistance to a similar anthracycline, daunorubicin (Lagadinou et al., 2008).

4.4.3.2 Vincristine

Collectively, the results presented in Section 4.3.3.3 suggest that inhibition of JNK signalling, using SP600125, protects U937 cells from vincristine-induced cell death. SP600125 and vincristine have previously been used in pre-B cell ALL cell lines and results showed that SP600125 did not alter the sensitivity of the cells to vincristine (Saunders, Cisterne, Weiss, Bradstock, & Bendall, 2011).

In contrast to SP600125, JNK-IN-8 did not appear to provide any protection against the metabolic effects of vincristine (Section 4.3.3.4). JNK-IN-8 however provided some protection against vincristine-induced necrosis. The conclusions which can be drawn from the JNK-IN-8 and vincristine data are limited and further experiments are required to confirm the effects of JNK-

IN-8 and vincristine on U937 cell death. Similarly to doxorubicin, this study is limited in the fact that the effects of JNK inhibition and vincristine treatment on the activation of JNK signalling components was not tested. Whilst vincristine-induced activation of JNK has been reported in other cancer cells such as human epidermal carcinoma cells (Stone & Chambers, 2000), breast cancer cells (Wang et al., 1998) and B-cell lymphoma cells (Muscarella & Bloom, 2008), the activation in leukaemia cells is less well reported. There appears to be no published reports of the effect of vincristine on JNK activation, specifically in U937 cells but JNK is activated in an alternative human leukemic monocyte cell line, THP-1 cells (Yoshida et al., 2011). JNK activation following vincristine treatment has also been reported *ex vivo* and *in vivo* in lymphocytes isolated from CLL patients (Bates, Lewis, Eastman, & Danilov, 2015).

4.4.3 JNK inhibition and heat treatment

Inhibition of JNK signalling, using SP600125 prior to heat treatment, had minimal effect on cell death after 24 h and 48 h (Section 4.4.4.1). This suggests JNK signalling does not have an important role in the response of U937 cells to heat treatment. In contrast, inhibition of JNK signalling, using JNK-IN-8, made U937 cells more susceptible to heat treatment-induced cell death after 24 and 48 h (Section 4.4.4.2).

Therefore there appears to be a difference in the ability of SP600125 and JNK-IN-8 to alter the susceptibility of U937 cells to heat-induced cell death. Heat shock proteins (HSPs) are able to protect cells from apoptosis caused by various cell stressors, including heat treatment (Creagh, Sheehan, & Cotter, 2000; Kennedy, Jäger, Mosser, & Samali, 2014). It has been reported that HSPs, in particular HSPA1A provides protection against apoptosis by preventing the activation of JNK (Gabai et al., 1997; Mosser et al., 1997; Volloch, Mosser, Massie, & Sherman, 1998). SP600125 also prevents the activation of JNK signalling and it is therefore possible that SP600125 has no effect on heat-induced cell death as JNK activation is already supressed by heat shock proteins. The effects of SP600125 and heat treatment on heat shock protein expression and JNK activation however is required to confirm the involvement of JNK activation in heat-induced cell death in U938 cells.

The observation that JNK-IN-8 makes U937 cells susceptible to heat-induced apoptosis suggests the importance of c-Jun phosphorylation in providing protection against heat-induced cell death. c-Jun is a downstream target of JNK activation, however as inhibition of JNK activation using SP600125 had minimal effect on heat-induced cell death, it is likely that the activation of c-Jun in response to heat-treatment is mediated by an alternative pathway to JNK signalling. c-Jun is also a downstream target of ERK signalling (Cargnello & Roux, 2011; Leppä, Saffrich, Ansorge, & Bohmann, 1998), therefore it is possible that the activation of c-Jun mediated by an alternative pathway such as ERK signalling, is important for mediating cell survival in U937 cells in response to heat treatment. Cross-talk between signalling pathways will be discussed further in Chapter 8.

4.5 Summary

The results presented in this chapter conclude that both SP600125 and JNK-IN-8 are effective at inhibiting JNK signalling in U937 cells. Although SP600125 did not prevent the phosphorylation of JNK as expected, JNK signalling inhibition by both inhibitors was evident by the decreased phosphorylation of the downstream target c-Jun.

Whilst it has been established that JNK signalling is important for the transforming ability and survival of BCR/ABL-expressing CML cells (Dickens et al., 1997; Hess et al., 2002; Raitano et al., 1995) the role of JNK in the survival of other types of leukemic cells is not currently understood. The results presented in this chapter show inhibition of JNK signalling using SP600125 sensitised U937 cells to UV-induced apoptosis, suggesting an important role of JNK signalling in mediating cell survival in response to UV, however inhibition of JNK using JNK-IN-8 did not alter the sensitivity of U937 cells to UV-induced apoptosis. Inhibition of JNK signalling, using JNK-IN-8 showed activation of c-Jun is important for mediating U937 cell survival in response to heat treatment, but this is likely to be controlled by a pathway other than JNK signalling as inhibition of JNK signalling using SP600125 had minimal effects on heat-induced cell death. Both JNK inhibitors, SP600125 and JNK-IN-8 provided protection against doxorubicin and vincristine-induced cell death.

These results suggest that JNK signalling is required for U937 cell death-induced by the chemotherapeutic agents vincristine and doxorubicin.

Chapter 5

Targeting p38 signalling in U937 cell line

Chapter 5: Targeting p38 signalling in U937 cell line

5.1 Introduction

In the previous chapter, the JNK signalling pathway was targeted in U937 cells in order to gain a greater understanding of the role of MAPK signalling in leukemic cell death. This chapter investigates the role of an alternative MAPK signalling pathway, the p38 pathway, which is activated by similar stimuli to the JNK signalling pathway.

p38 signalling has been implicated in leukemogenesis; p38 signalling mediates the interaction between leukemic progenitors and the bone marrow stroma, which is required for the migration and survival of leukemic cells (Bendall et al., 2005; Gaundar et al., 2009; Juarez et al., 2009; Ringshausen et al., 2004). These studies however implicate the role of p38 in the development of the lymphocytic leukaemias, ALL and CLL. The role of p38 signalling in the development and progression of myeloid leukaemias however remains poorly understood. Although it has been shown that p38 signalling is required for TNF-α-mediated survival and proliferation of AML cells (Liu, Fan, Liu, Olashaw, & Zuckerman, 2000), further research is needed to gain a more thorough understanding of the role of p38 signalling in the survival of myeloid leukemic cells.

The p38 signalling pathway is activated by stress stimuli, including chemotherapy drugs. It is therefore not surprising that activation of p38 is a common event following the treatment of leukemic cells with chemotherapeutic agents including: arsenic trioxide, interferon-α, rituximab and dasatinib (Dumka et al., 2009; Giafis et al., 2006; Maha et al., 2009; Mayer et al., 2001; Pedersen et al., 2002). The U937 model cell line will be used in this chapter to investigate the role of p38 signalling in the response of AML cells to chemotherapy. p38 activation has previously been shown to be essential for the induction of AML cell death in response to chemotherapeutic agents such as all-*trans*-retinoic-acid, arsenic trioxide, cytarabine and daunorubicin (Alsayed et al., 2001; Giafis et al., 2006; Maha et al., 2009). Inhibition of p38 signalling prevents cell death induced by these chemotherapeutic agents. Development of novel therapies to enhance p38 activation in response to chemotherapy may be an approach to induce cell

death in leukemic cells. However in order for this to be successful, it is necessary to understand the role of p38 signalling in the response to specific chemotherapeutic agents; in this chapter the role of p38 in the response to vincristine and doxorubicin will be investigated.

Aims

The overall aim of this chapter is to gain a greater understanding of the role of p38 signalling in leukemic cell death using the model U937 cell line. This will be achieved by:

- targeting p38 signalling using the small-molecule p38 inhibitors SB202190 and SB203580;
- determining the effects of p38 signalling inhibition on the susceptibility of U937 cells to various cell stressors (UV light, chemotherapeutic agents and heat treatment).

A similar approach to that taken in Chapter 4 for JNK signalling will be taken in this chapter to investigate the role of p38 signalling in leukemic cell death. To gain a thorough understanding of the role of p38 signalling two inhibitors, SB202190 and SB203580, will be utilised. The effect of SB202190 and SB203580 on cell death and the activation of p38 signalling components will initially be tested. Following this, the inhibitors will each be used in combination with a range of cell stressors, and the effect on U937 cell death will be measured using multiple techniques.

5.2 Methods

5.2.1 Cell culturing

U937 cell line was cultured and maintained as described in Section 2.1.1. Prior to treatments, cells were prepared as described in Section 2.1.4.

5.2.2 Treatment of U937 cells with p38 inhibitors

The p38 inhibitors, SB202190 and SB203580, prepared as described in Section 2.2.1, were used in this chapter. U937 cells were treated with SB202190 and SB203580 as described in Section 2.2.1.

5.2.3 Measurement of the effects of p38 inhibition on U937 cell death

The effect of p38 inhibition on U937 cell death was assessed using:

- MTS assay to measure metabolic activity (Section 2.4.1.3)
- PI assay to measure necrosis (Section 2.4.1.4)

5.2.4 Treatment of U937 cells with p38 inhibitor and anisomycin

U937 cells were treated with the p38 inhibitors and anisomycin as described in Section 2.2.

5.2.5 Measurement of the effects of p38 inhibition on MAPK activation

The effect of p38 inhibition and anisomycin on the phosphorylation of p38 was measured by:

- Western blotting (Section 2.3.1)
- flow cytometry (Section 2.3.2)

5.2.6 Treatment of U937 cells with p38 inhibitors and cell stressors

U937 cells were treated with p38 inhibitors in combination with cell stressors, as described in Section 2.2.

5.2.7 Measurement of the effects of p38 inhibition and cell stressors on U937 cell death

The effect of p38 inhibition and cell stressors on U937 cell viability was assessed using:

- MTS assay to measure metabolic activity (Section 2.4.1.3)
- PI assay to measure necrosis (Section 2.4.1.4)
- FITC Annexin V FITC Apoptosis Detection Kit I to measure cell viability (Section 2.4.2.1)
- SensoLyte® Homogeneous Rh110 Caspase-3/7 Assay Kit to measure active caspase 3/7 levels (Section 2.4.1.5)
- microscopy to determine changes in cell morphology (Section 2.5)

5.3 Results

5.3.1 Inhibition of p38 signalling

5.3.1.1 Effect of SB202190 on U937 cell death

In order to determine the effect of SB202190 on U937 cell death, the dose-dependent effect of SB202190 on U937 metabolic activity was initially assessed. Treatment with 12.5 µM or less SB202190 did not cause a decrease in metabolic activity compared to untreated cells (Figure 5.1A). Metabolic activities were significantly reduced ($P<0.001$) following treatment with 25 to 100 µM SP600125.

In order to establish if the observed reductions in metabolic activity were due to cell death by necrosis, a PI assay was performed. Treatment with 50 and 100 µM SB202190 resulted in a significant ($P<0.0001$) increase in the percentage of PI-positive cells, however treatment with 25 µM SB202190 or less had no significant effect on U937 necrosis (Figure 5.1B).

The effect of 0 to 25 µM SB202190 on U937 cell death was investigated further by measuring annexin V binding and PI staining on the flow cytometer. Results confirmed concentrations of SB202190, 25 µM or less, had no significant effect on U937 cell viability (Figure 5.1C) and hence no effect on apoptosis (Figure 5.1D). Collectively, these results show 25 µM or less of SB202190 does not induce cell death in U937 cells and therefore these concentrations were selected to investigate the effect on p38 signalling.

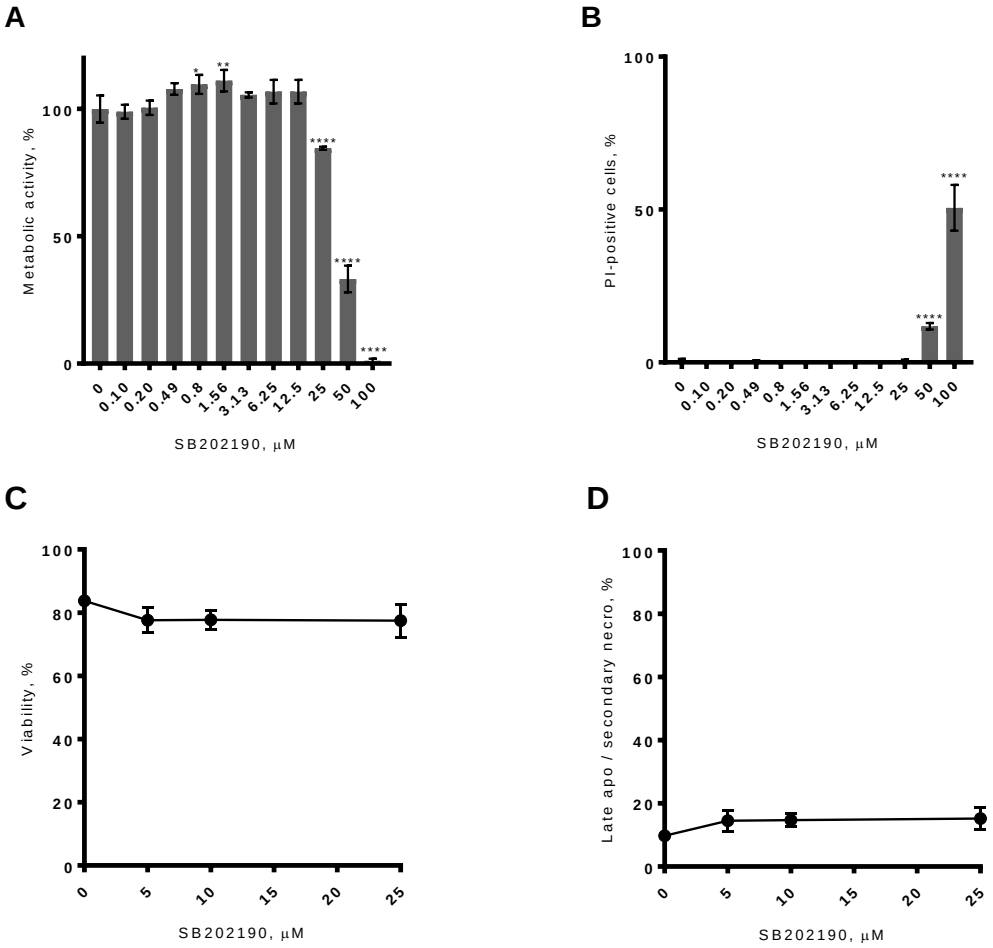

Figure 5.1: Effect of SB202190 on U937 cell death
U937 cells were treated with various concentrations of SB202190 and then incubated at 37°C for 24 h before an (A) MTS assay, (B) PI assay or (C,D) BD Pharmingen™ FITC Annexin V Apoptosis Detection Kit I were performed. Data presented as mean ± SD; n=3. Statistical differences shown to untreated cells, calculated using one-way ANOVA with Dunnett's *post hoc* test: * (P<0.05), ** (P<0.01), *** (P<0.001), **** (P<0.0001).

5.3.1.2 Effect of SB202190 on MAPK phosphorylation

The effect of 0 to 25 µM SB202190 on MAPK phosphorylation was measured by flow cytometry and Western blotting. Pre-treatment with 3 µM SB202190 had no significant effect on the percentage of cells expressing phosphorylated p38, compared to anisomycin-only treated cells (Figure 5.2A). Although phosphorylated-p38 MFI was significantly ($P<0.01$) reduced following treatment with 3 µM SB202190, the effects were minimal (Figure 5.2B). The percentages of cells expressing phosphorylated p38 were significantly reduced ($P<0.0001$) following pre-treatment with 12.5 and 25 µM SB202190 (Figure 5.2A). Phospho-p38 MFIs were also significantly ($P<0.01$) decreased for cells pre-treated with 12.5 and 25 µM SB202190 (Figure 5.2B). These results showed 12.5 and 25 µM SB202190 can inhibit p38 phosphorylation. Western blotting confirmed 10 µM SB202190 inhibited p38 phosphorylation, and that this inhibition was not complete (Figure 5.2C).

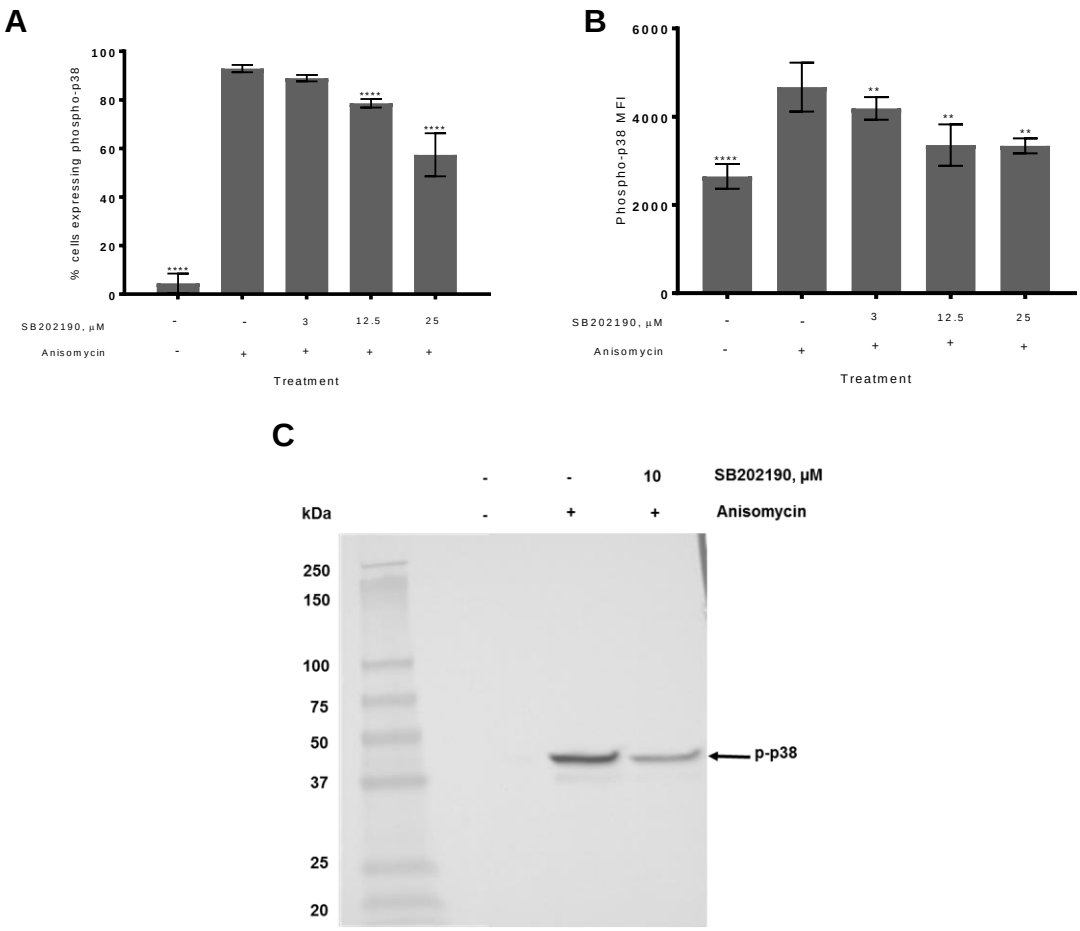

Figure 5.2: Effect of SB202190 and anisomycin on p38 phosphorylation
U937 cells were pre-treated with various concentrations of SB202190 at 37ºC for 1 h and then incubated with 1 µM anisomycin at 37ºC for 30 min. Cells were then prepared for (A,B) flow cytometry or (C) Western blotting. (A,B) Cells were fixed with 4% paraformaldehyde, permeabilised with 90% ice-cold methanol and stained with phospho-p38 (Thr180/Tyr182) Alexa Fluor 647-conjugated antibody. (A) shows the percentage of cells expressing phospho-p38 and (B) shows phospho-p38 mean fluorescence intensity (MFI). Data presented as mean ± SD, n=3. Statistical differences shown to anisomycin-only treated cells, calculated using one-way ANOVA with Dunnett's post hoc test: ** (P<0.01), **** (P<0.0001). (C) Total protein was extracted and separated with SDS-PAGE electrophoresis using 4-15% precast gels. Proteins were electro-transferred to nitrocellulose membrane and phospho-p38 was detected by Western blotting using phospho-p38 (Thr180/Tyr182) and anti-rabbit peroxidase.

5.3.1.3 Effect of SB203580 on U937 cell death

The effect of SB023580, an alternative p38 inhibitor, on U937 cell death was investigated. Assessment of the dose-dependent effect of SB203580 on U937 metabolic activity showed treatment with up 25 µM SB202190 had no significant effect on metabolic activity compared to untreated cells (Figure 5.3A). Metabolic activity was significantly ($P<0.001$) reduced following treatment with 50 and 100 µM SB203580. Treatment with 100 µM SB203580 also caused a significant ($P<0.0001$) increase in the percentage of PI-positive cells (Figure 5.3B).

Flow cytometry, assessing cell viability by measuring annexin V binding and PI staining, confirmed that treatment with 25 µM or less SB203580, had no significant effect on U937 cell viability and hence no effect on apoptosis (Figures 5.3C-D)

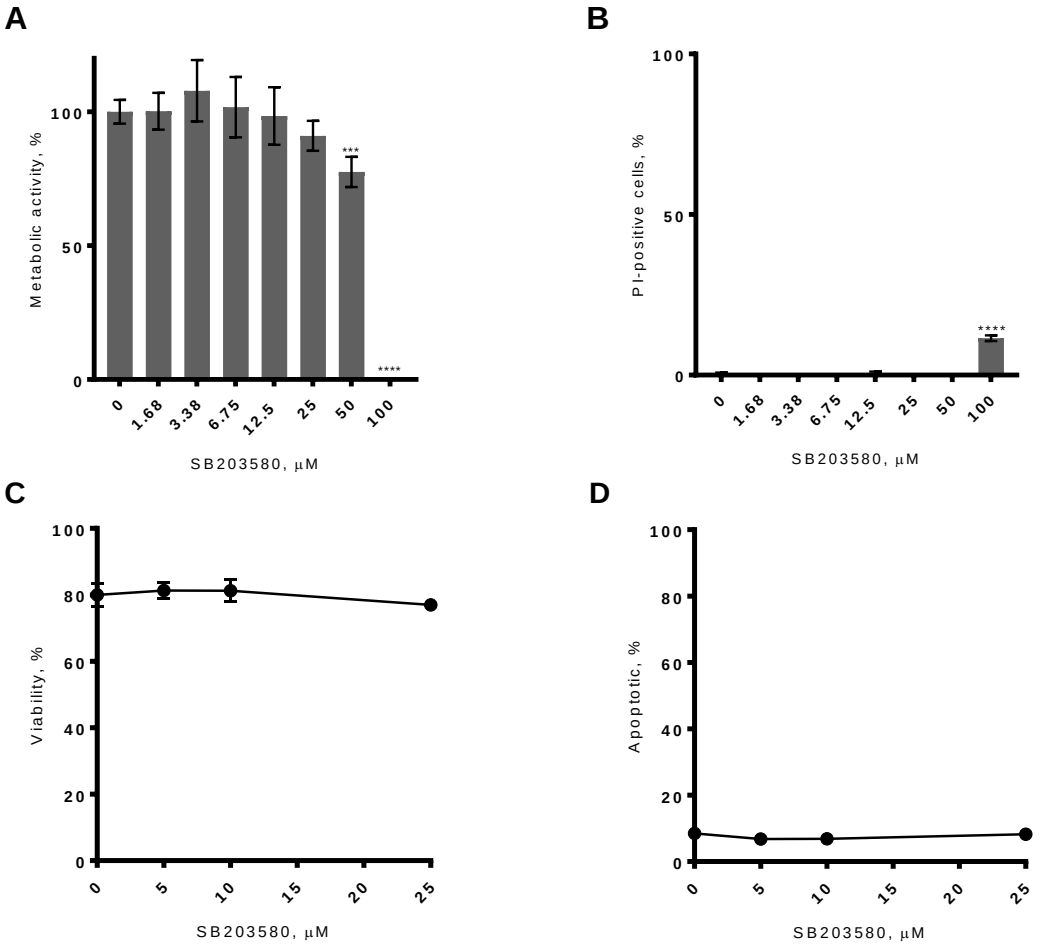

Figure 5.3: Effect of SB203580 on U937 cell death
U937 cells were treated with various concentrations of SB203580 and then incubated at 37ºC for 24 h before an (A) MTS assay, (B) PI assay or (C,D) BD Pharmingen™ FITC Annexin V Apoptosis Detection Kit I were performed. Data presented as mean ± SD; n=3. Statistical differences shown to untreated cells, calculated using one-way ANOVA with Dunnett's *post hoc* test: *** (P<0.001), **** (P<0.0001).

5.3.1.4 Effect of SB203580 on MAPK phosphorylation

As 0 to 25 µM SB203580 had no effect on U937 cell death, the effect of concentrations of SB203580 up to 25 µM, on p38 phosphorylation, was assessed. Treatment with 3 to 25 µM SB203580 caused significant ($P<0.05$) decreases in the percentages of cells expressing phosphorylated p38, compared to anisomycin-only treated cells (Figure 5.4A). Although statistically significant, the reductions were minimal; the difference to untreated cells was only around 10%. Phospho-p38 MFIs were significantly ($P<0.001$) lower for cells pre-treated with 3 to 25 µM SB203580 prior to anisomycin treatment, compared to anisomycin-only treated cells, but the differences in phospho-p38 MFIs were also minimal (Figure 5.4B). Western blotting showed that p38 phosphorylation induced by anisomycin treatment, was not inhibited by 3 to 25 µM SB203580 (Figure 5.4C).

To further investigate the effect of SB203580 on p38 signalling, the effect of SB203580 on MAPKAPK-2 phosphorylation, a downstream target of p38, was measured by Western blotting (Figure 5.4D). The bands of phosphorylated MAPKAPK-2 however were not clear. This, combined with the lack of a total MAPKAPK-2 blot limits the interpretation of these results. While no band corresponding to MAPKAPK-2 was detected in untreated U937 cells, a faint signal at <50 kDa was detected following anisomycin treatment. Due to the weakness of this signal, it is unclear if 10 µM SB203580 inhibited MAPKAPK-2 phosphorylation.

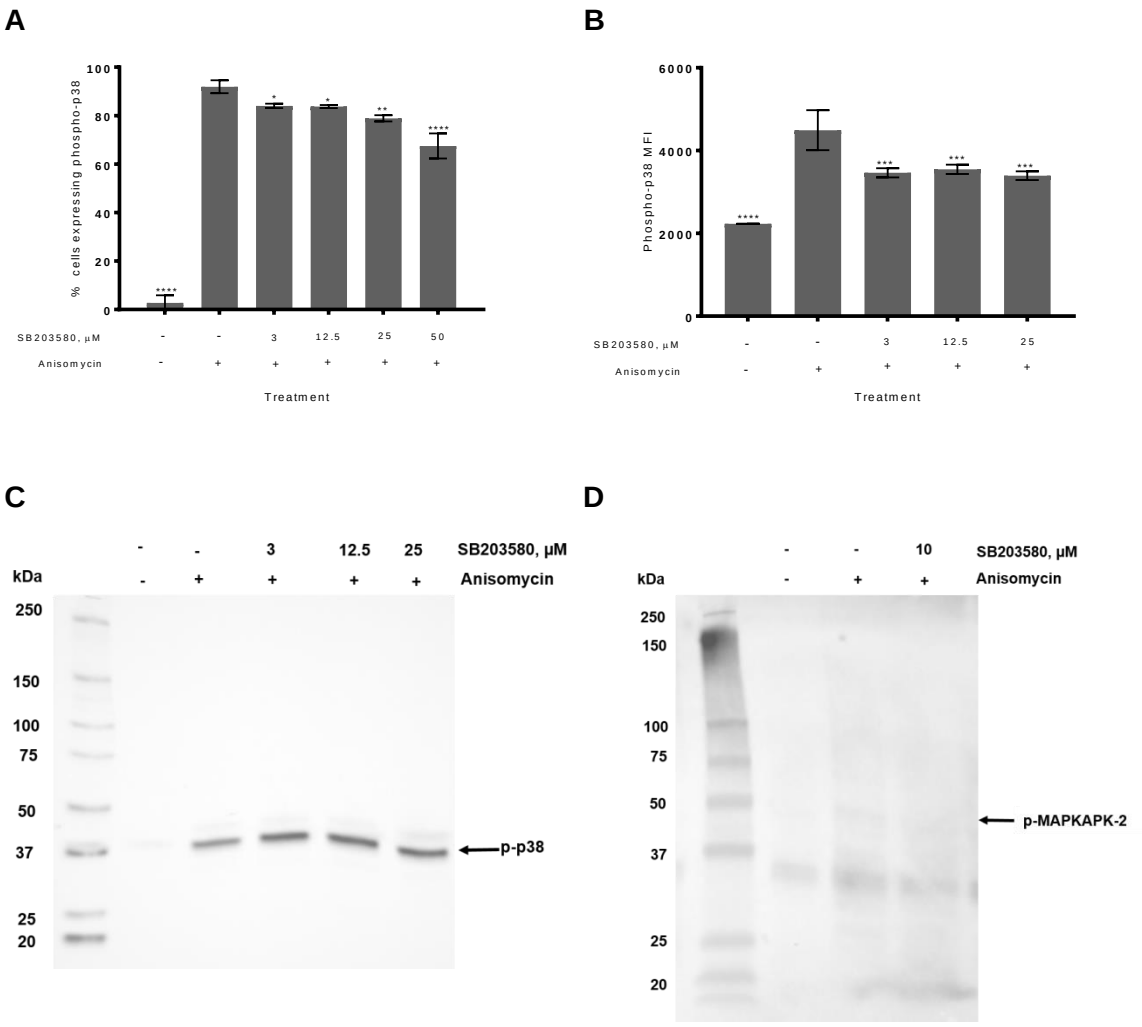

Figure 5.4: Effect of SB203580 and anisomycin on MAPK phosphorylation
U937 cells were pre-treated with various concentrations of SB203580 at 37°C for 1 h and then incubated with 1 µM anisomycin at 37°C for 30 min. Cells were then prepared for (A,B) flow cytometry or (C,D) Western blotting. (A,B) Cells were fixed with 4% paraformaldehyde, permeabilised with 90% ice-cold methanol and stained with phospho-p38 (Thr180/Tyr182) Alexa Fluor 647-conjugated antibody. (A) shows the percentage of cells expressing phospho-p38 and (B) shows phospho-p38 mean fluorescence intensity (MFI). Data presented as mean ± SD, n=3. Statistical differences shown to anisomycin-only treated cells, calculated using one-way ANOVA with Dunnett's *post hoc* test: * (P<0.05), ** (P<0.01), *** (P<0.001), **** (P<0.0001). (C,D) Total protein was extracted and separated with SDS-PAGE electrophoresis using 4-15% precast gels. Proteins were electro-transferred to nitrocellulose membrane and (C) phospho-p38 was detected by Western blotting using phospho-p38 (Thr180/Tyr182) and anti-rabbit peroxidase or (D) phospho-MAPKAPK-2 was detected by Western blotting using phospho-MAPKAPK-2 (Thr222) and anti-rabbit peroxidase.

5.3.2 Effect of p38 inhibition and UV light

5.3.2.1 Effect of SB202190 and UV light on U937 cell death

To investigate the role of JNK signalling in UV-induced cell death, U937 cells were treated with 10 µM SB202190 prior to treatment with various durations of UV light. For cells treated with 15 to 30 s UV, metabolic activities were significantly ($P<0.0001$) lower for cells pre-treated with 10 µM SB202190 compared to those not pre-treated with SB202190 (Figure 5.5A). Results of the PI assay showed SB202190 had no significant effect on the percentages of PI-positive cells (Figure 5.5B) and therefore the differences observed in metabolic activities (Figure 5.5A) were not due to necrosis.

Flow cytometry results confirmed inhibition of p38 signalling by SB202190 increased the susceptibility of U937 cells to UV-induced cell death. For cells treated with 10 and 15 s UV, cell viability was significantly ($P<0.001$) lower for cells pre-treated with SB202190 compared to cells not treated with SB202190 (Figure 5.5.C). These differences were not due to early apoptotic cells (Figure 5.5D) but were due to significant ($P<0.001$) increases in the percentages of late apoptotic/secondary necrotic cells (Figure 5.5.E).

Microscopy images provided further confirmation of the effects of SB202190 and UV on U937 cell death (Figure 5.5F). In the absence of UV light, cells not pre-treated with SB202190 appeared healthy and did not stain with PI, whereas whilst the majority of cells pre-treated with SB202190 also appeared healthy, a few were misshapen and stained with PI. Some of the cells treated with 9 s UV, but not pre-treated with SB202190, appeared apoptotic and stained with PI, however, a small number of cells appeared viable. For cells treated with 10 µM SB202190 prior to UV light treatment, all cells appeared apoptotic and the majority stained with PI and showed fragmented nuclei. Taken together, these results show that inhibition of p38 signalling using SB202190 increases the susceptibility of U937 cells to UV-induced cell death.

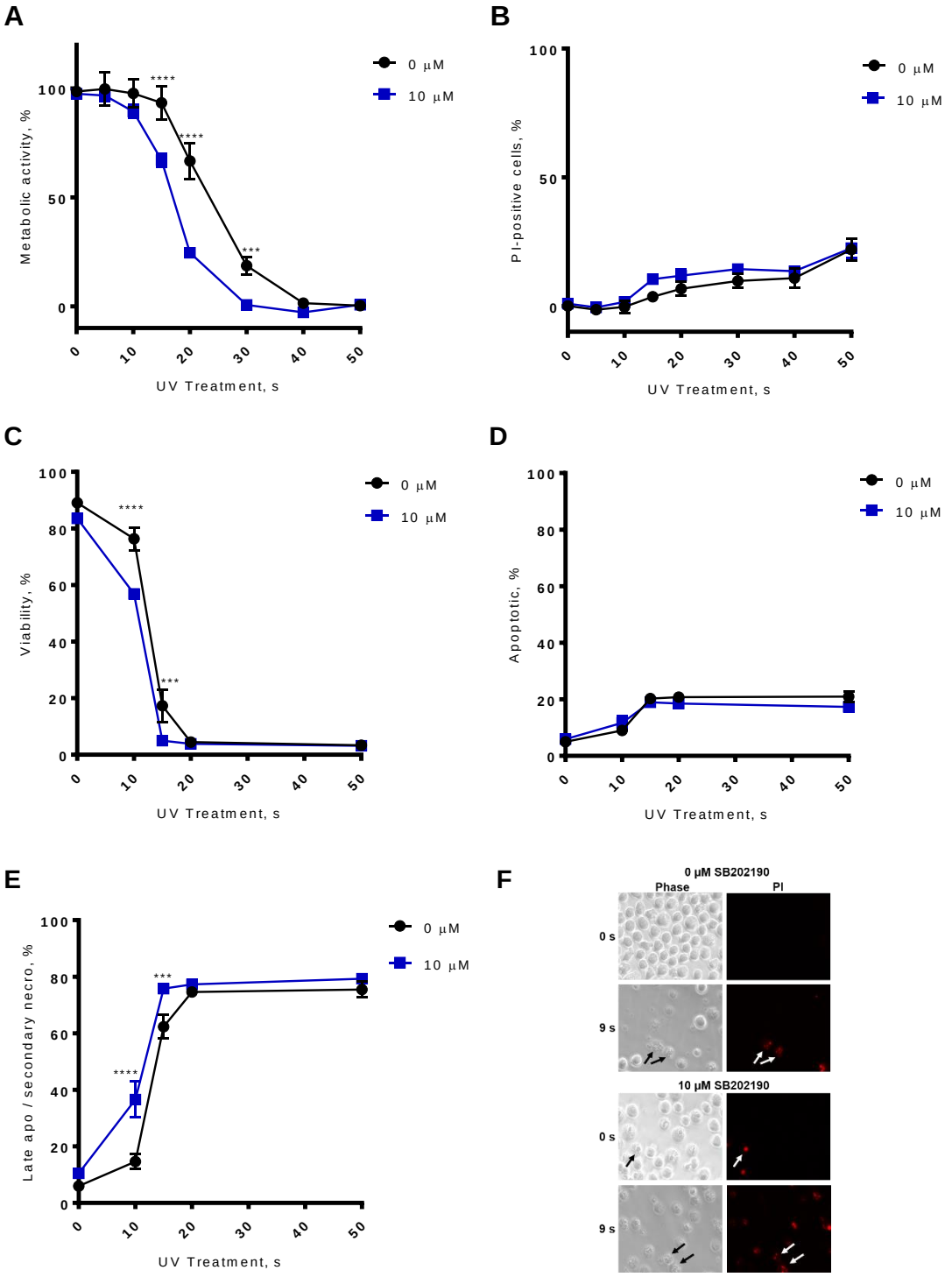

Figure 5.5: Effect of SB202190 and UV light treatment on U937 cell death
U937 cells were pre-treated with 0, 10 or 25 µM SB202190 at 37°C for 1 h to inhibit p38 signalling. Cells were then treated with various durations of UV light. After 24 h incubation at 37°C, an (A) MTS assay, (B) PI assay, (C-E) BD Pharmingen™ FITC Annexin V Apoptosis Detection Kit I or (F) microscopy were performed. Data presented as mean ± SD; n=3. Statistical differences shown between SB202190 and no inhibitor at UV time points, calculated using two-way ANOVA with Sidak's *post hoc* test: *** (P<0.001), **** (P<0.0001). (F) Cells were stained with PI staining solution and visualised using EVOS™FL AutoCell Imaging System at 40 X magnification. Arrows indicate apoptotic cells. Images shown are representative of 3 individual images collected.

5.3.2.2 Effect of SB203580 and UV light on U937 cell death

To further investigate the role of p38 signalling in UV-induced cell death, U937 cells were pre-treated with SB203580 prior to treatment with various durations of UV light. Due to the inconclusive data showing the ability of SB203580 to inhibit p38 signalling, the effect of both 10 and 25 µM SB203580 were investigated. For cells treated with 15 to 40 s UV, pre-treatment with either 10 or 25 µM SB203580, caused significant ($P<0.05$) reductions in metabolic activities compared to cells treated without SB203580 (Figure 5.6A and Table 5.1). There were no significant differences in necrosis between SB203580-treated cells and those treated without SB203580, when treated with 0 to 10 s UV light (Figure 5.6B and Table 5.1). For 15 to 50 s UV, there were significantly ($P<0.05$) more PI-positive cells for SB203580-treated cells compared to cells not treated with SB203580, when treated with equivalent durations of UV light. The differences in metabolic activities and the percentages of PI-positive cells were however minimal.

Flow cytometry results showed that cell viability was only significantly ($P<0.0001$) lower for cells pre-treated with SB203580 compared to cells not treated with SB203580, when treated with 6 s UV light (Figure 5.7A). For other durations of UV light, no significant differences in viabilities were measured as the percentages of apoptotic cells were higher (Figure 5.7B), but the percentages of late apoptotic cells were lower (Figure 5.7C), for cells not pre-treated with SB203580 compared to cells treated with SB203580.

Microscopy images also indicated that pre-treating cells with SB203580 prior to UV treatment resulted in increased cell death (Figure 5.7D). For cells pre-treated with SB203580 prior to 6 s UV light, a greater number of cells appeared apoptotic and the majority stained with PI and showed fragmented nuclei. Taken together, the results indicate that SB203580 can increase the susceptibility of U937 cells to UV, however the effect is minimal.

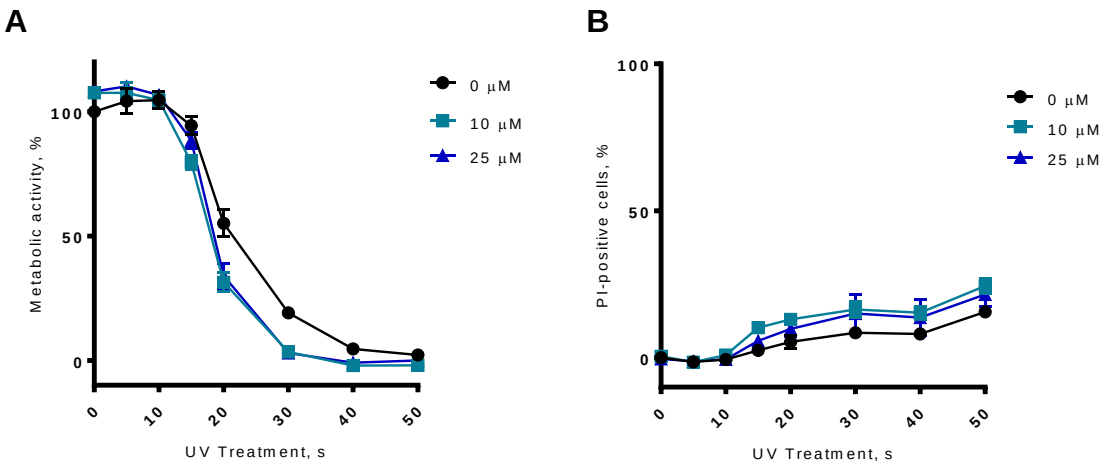

Figure 5.6: Effect of SB203580 and UV light treatment on U937 metabolic activity and necrosis, measured by MTS and PI assays
U937 cells were pre-treated with 0, 10 or 25 µM SB203580 at 37°C for 1 h to inhibit p38 signalling and then treated with various durations of UV light. After 24 h incubation at 37°C, an (A) MTS assay and (B) PI assay were performed. Data presented as mean ± SD; n=4.

Table 5.1: Statistical analysis to show the effects of SB203580 and UV light treatment on U937 metabolic activity and necrosis
Data presented as mean ± SD; n=4. Statistical differences shown between SB203580 and no inhibitor at UV time points, calculated using two-way ANOVA with Tukey's *post hoc* test: * (P<0.05), ** (P<0.01), *** (P<0.001), **** (P<0.0001).

MTS assay		
UV Treatment, s	**SB203580 concentration**	
	0 µM vs 10 µM	**0 µM vs 25 µM**
0	**	***
5	ns	ns
10	ns	ns
15	****	*
20	****	****
30	****	****
40	**	*
50	ns	ns
PI assay		
UV Treatment, s	**SB203580 concentration**	
	0 µM vs 10 µM	**0 µM vs 25 µM**
0	ns	ns
5	ns	ns
10	ns	ns
15	***	ns
20	***	*
30	****	**
40	***	**
50	****	**

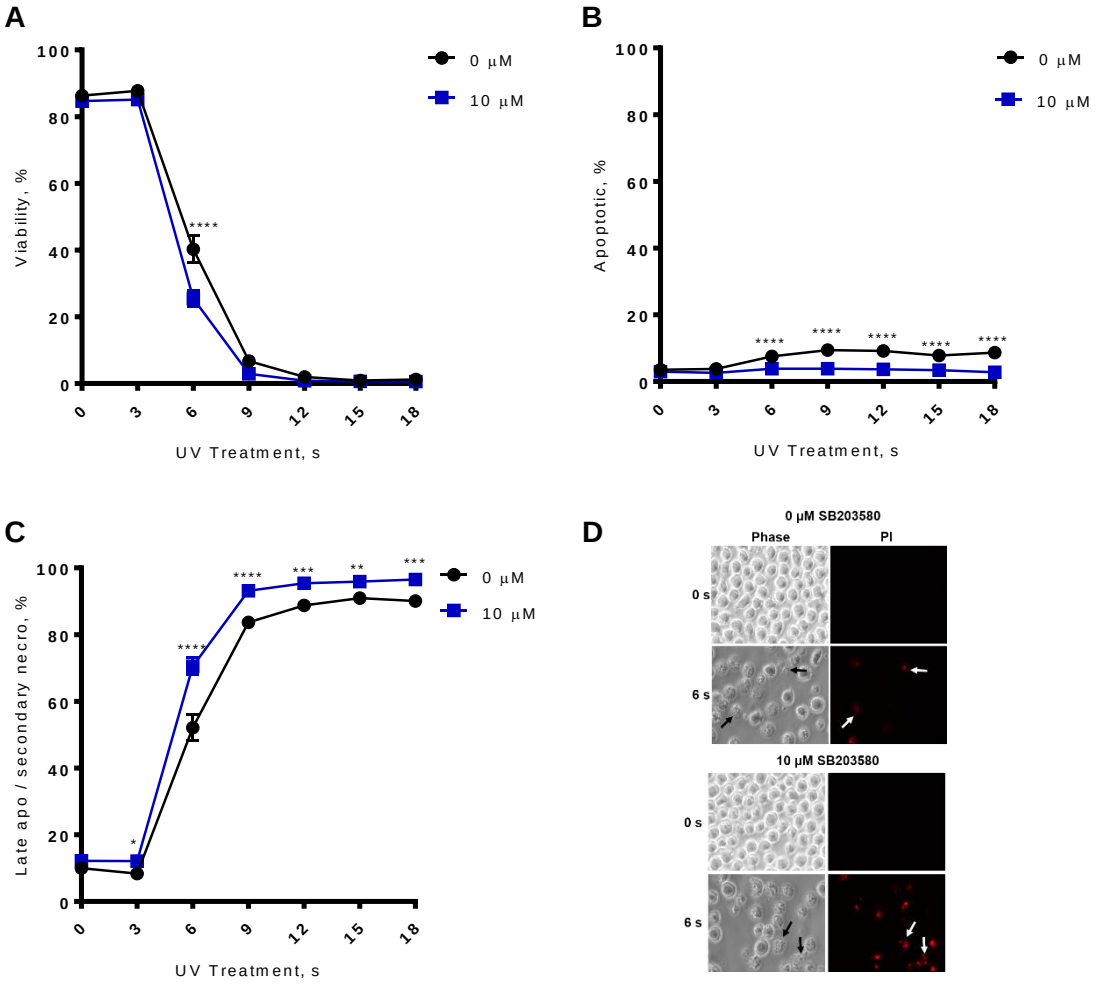

Figure 5.7: Effect of SB203580 and UV light treatment on U937 cell death
U937 cells were pre-treated with 0 or 10 µM SB203580 at 37°C for 1 h to inhibit p38 signalling and then treated with various durations of UV light. After 24 h, (A) cell viability, (B) apoptosis and (C) late apoptosis/secondary necrosis were measured using BD Pharmingen™ FITC Annexin V Apoptosis Detection Kit Ior (D) microscopy was performed. Data presented as mean ± SD, n=3. Statistical differences shown between SB203580 and no inhibitor at UV time points, calculated using two-way ANOVA with Sidak's *post hoc* test: * (P<0.05), ** (P<0.01), *** (P<0.001), **** (P<0.0001). (D) Cells were stained with PI staining solution and visualised using EVOS™FL AutoCell Imaging System at 40 X magnification. Arrows indicate apoptotic cells. Images shown are representative of 3 individual images collected.

5.3.3 Effect of p38 inhibition and chemotherapy

5.3.3.1 Effect of SB202190 and doxorubicin on U937 cell death

The role of p38 signalling in doxorubicin-induced cell death was initially assessed by measuring metabolic activity following SB202190 and doxorubicin treatment. For cells treated with 0 to 0.8 doxorubicin, there were no significant differences in metabolic activities between cells pre-treated with and without SB202190 (Figure 5.8A). For cells treated with 1.56 to 50 µM doxorubicin, metabolic activities were significantly ($P<0.01$) lower for cells pre-treated with SB2021290 compared to cells not pre-treated with SB202190, when treated with equivalent concentrations of doxorubicin.

To determine if the observed differences in metabolic activities were due to differences in apoptosis, active caspase-3/7 levels were measured following SB202190 and doxorubicin treatment. For cells treated with 0 to 15 µM doxorubicin, no significant differences in active caspase-3/7 levels were measured between cells treated with SB202190 and cells not treated with SB202190 (Figure 5.8B). Active caspase levels were significantly ($P<0.0001$) higher for U937 cells pre-treated with SB202190 compared to cells not treated with SB202190, when treated with 25 µM doxorubicin; however, the levels were only around 2-fold higher. It is possible that 9 h was not the most appropriate time point to measure caspase-3/7 activation.

For cells treated with 10 and 25 µM doxorubicin, there did not appear to be any morphological differences in U937 cells, between cells pre-treated with and without SB202190 (Figure 5.8C). All doxorubicin-treated cells appeared apoptotic; cells appeared misshapen and some, but not all cells stained with DAPI. Taken together, there is limited evidence to suggest the direct role of p38 in doxorubicin-induced cell death in U937 cells.

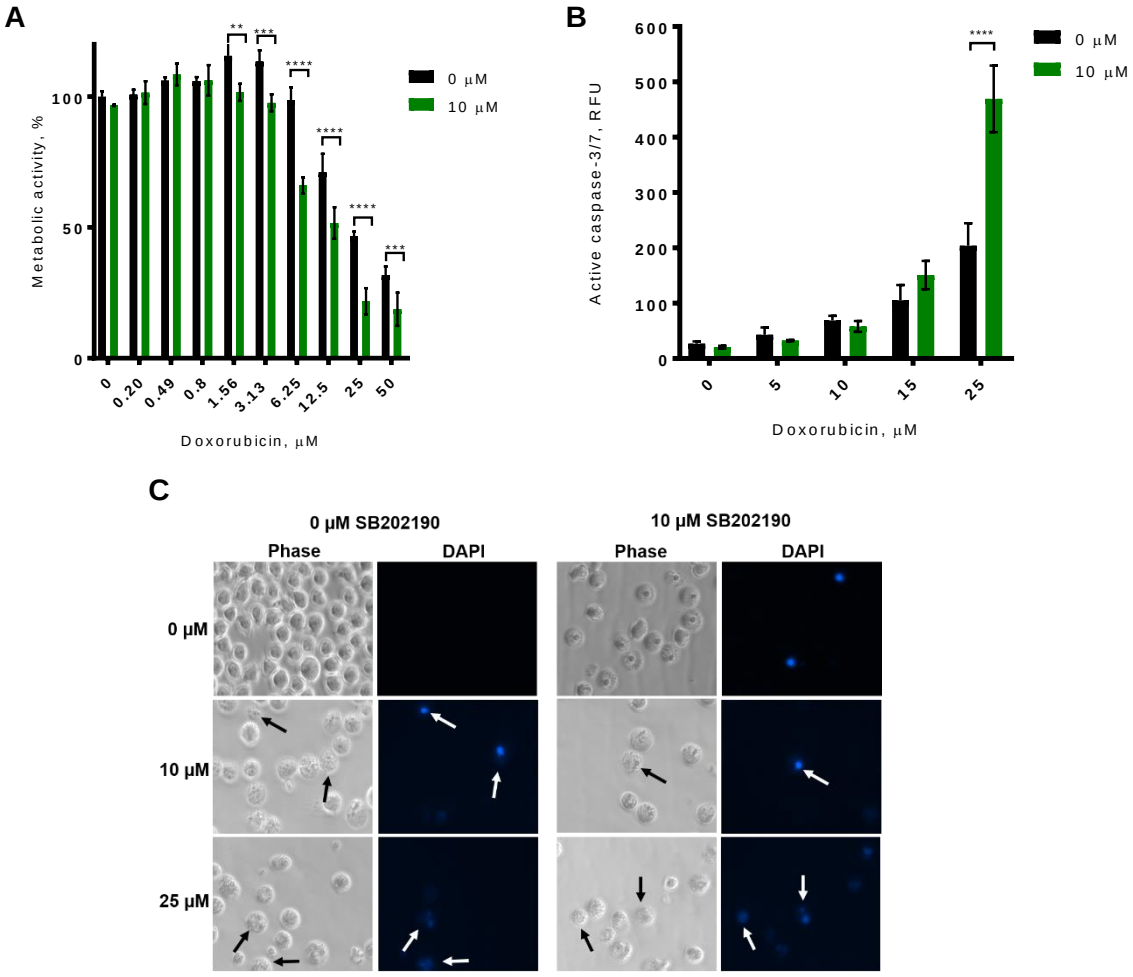

Figure 5.8: Effect of SB202190 and doxorubicin on U937 cell death
U937 cells were pre-treated with 0 or 10 µM SB202190 at 37°C for 1 h to inhibit p38 signalling and then treated with various concentrations of doxorubicin. Cells were then incubated at 37°C for (A) 24 h before an MTS assay or (B) 9h before active caspase-3/7 levels were measured using SensoLyte® Homogeneous Rh110 Caspase-3/7 Assay Kit. Statistical differences shown between SB2021900 and no inhibitor at doxorubicin concentration points, calculated using two-way ANOVA with Sidak's *post hoc* test: ** (P<0.01), *** (P<0.001), **** (P<0.0001). (C) Cells were stained with DAPI 24h after treatment and visualised using EVOS™FL AutoCell Imaging System at 40 X magnification. Black and white arrows indicate late apoptotic cells. Images shown are representative of 3 individual images collected

5.3.3.2 Effect of SB203580 and doxorubicin on U937 cell death

The effect of SB203580 and doxorubicin treatment on U937 metabolic activity was assessed and results showed no significant differences in metabolic activities between cells pre-treated with SB203580 and cells not pre-treated with SB203580, when treated with equivalent concentrations of doxorubicin, apart from 1.56 µM doxorubicin (Figure 5.9A). For all concentrations of doxorubicin tested, there were no significant differences in active caspase-3/7 levels between cells pre-treated with and without SB203580 (Figure 5.9B). These results confirm SB203580 has no significant effect on doxorubicin-induced cell death in U937 cells. This however could be due to the ineffectiveness of SB203580 to inhibit 38 signalling.

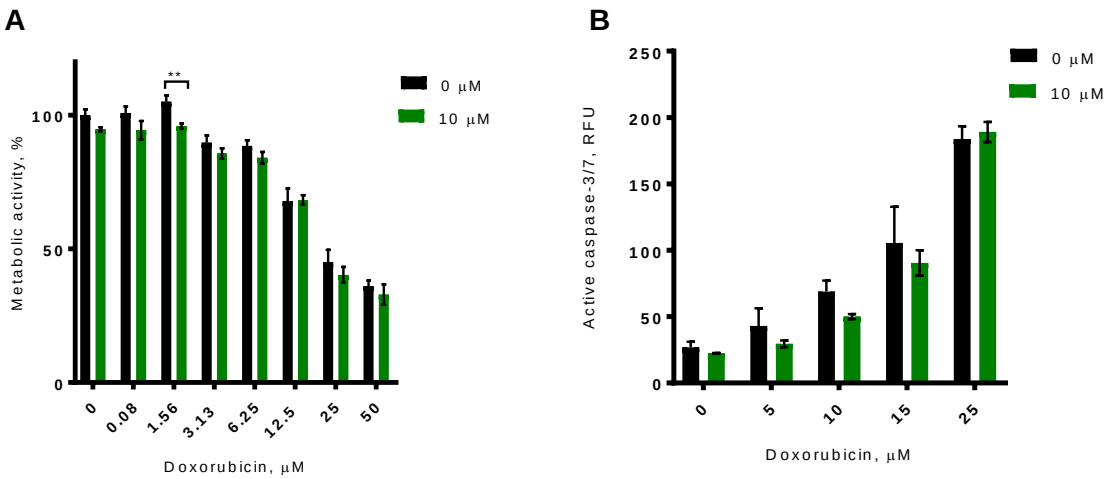

Figure 5.9: Effect of SB203580 and doxorubicin on U937 cell death
U937 cells were pre-treated with 0 or 10 µM SB203580 at 37°C for 1 h to inhibit p38 signalling and then treated with various concentrations of doxorubicin. Cells were then incubated at 37°C for (A) 24 h before an MTS assay was performed or (B) 9h before active caspase-3/7 levels were measured using SensoLyte® Homogeneous Rh110 Caspase-3/7 Assay Kit. Data presented as mean ± SD; n=3. Statistical differences shown between SB203580 and no inhibitor at doxorubicin concentration points, calculated using two-way ANOVA with Sidak's *post hoc* test: ** (P<0.01).

5.3.3.3 Effect of SB202190 and vincristine on U937 cell death

The role of p38 signalling in response to vincristine, an alternative chemotherapeutic agent was investigated. Initially the effect of SB202190 and vincristine treatment on U937 metabolic activity was measured. For U937 cells treated with 0.2 and 0.4 nM vincristine, metabolic activities were significantly ($P<0.01$) lower for cells pre-treated with SB202190 compared to cells not pre-treated with SB202190, when treated with equivalent concentrations of vincristine (Figure 5.10A). For U937 cells treated with 1.56 to 100 nM vincristine, metabolic activities for SB202190-treated cells were significantly ($P<0.01$) higher than cells not treated with SB202190, when treated with equivalent concentrations of vincristine. It was shown that the differences in metabolic activities were not due to cell death by necrosis (data not shown).

Assessment of cell viability, using flow cytometry, confirmed cell viabilities were significantly ($P<0.0001$) higher for cells pre-treated with SB202190 prior to vincristine treatment compared to cells not pre-treated with SB202190 (Figure 5.10B). The differences in cell viabilities were due mainly to lower percentages of apoptotic cells for cells pre-treated with SB202190, prior to vincristine treatment (Figure 5.10C), but also small differences in the percentages of late apoptotic/secondary necrotic cells (Figure 5.10D).

Fluorescence microscopy images were consistent with the results of the MTS assay and flow cytometry. For cells treated with 25 nM vincristine, without SB202190, the majority of cells appeared misshapen and PI staining showed fragmented nuclei (Figure 5.10D). For cells treated with SB202190 prior to vincristine, fewer cells appeared apoptotic and several cells which appeared healthy and did not stain with PI, were present. Taken together, these results suggest inhibiting p38 signalling using SB202190 provides U937 cells with protection against cell death.

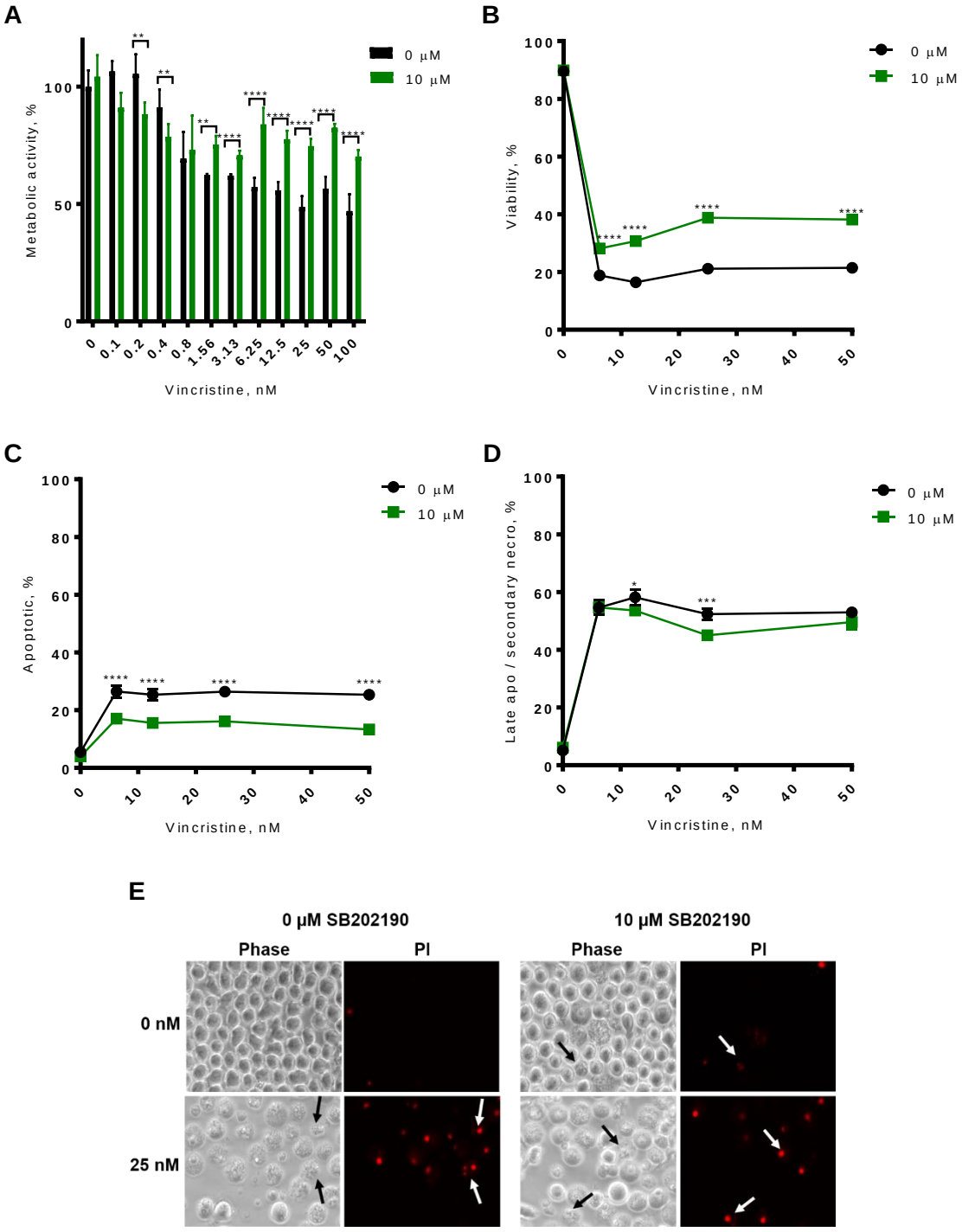

Figure 5.10: Effect of SB202190 and vincristine on U937 cell death
U937 cells were pre-treated with 0 or 10 µM SB202190 at 37°C for 1 h to inhibit p38 signalling and then treated with various concentrations of vincristine. Cells were then incubated at 37°C for 24 h before an A) MTS assay or (B-D) BD Pharmingen™ FITC Annexin V Apoptosis Detection Kit I or (E) microscopy were performed. Data presented as mean ± SD; n=3. Statistical differences shown between SB202190 and no inhibitor at vincristine concentration points, calculated using two-way ANOVA with Sidak's *post hoc* test: * (P<0.05), ** (P<0.01), *** (P<0.001), **** (P<0.0001). (E) Cells were stained with PI staining solution 24h after treatment and visualised using EVOS™FL AutoCell Imaging System at 40 X magnification. Black and white arrows indicate late apoptotic cells. Images shown are representative of 3 individual images collected

5.3.3.4 Effect of SB203580 and vincristine on U937 cell death

The role of p38 signalling in response to vincristine was investigated further utilising SB203580. Significant differences (P<0.05) between cells treated with and without SB203580 were only observed for cells treated with 0.1 and 0.8 nM (Figure 5.11A). The differences were however minimal; differences in metabolic activities for these concentrations were less than 10%. Results of the PI assay showed that for cells treated with 0.8 to 100 nM vincristine, the percentages of necrotic cells were significantly (P<0.05) higher for cells pre-treated with SB203580 (Figure 5.11B). Although significant, the differences were less than 5%. SB2023580 therefore has minimal effect on vincristine-induced cell death.

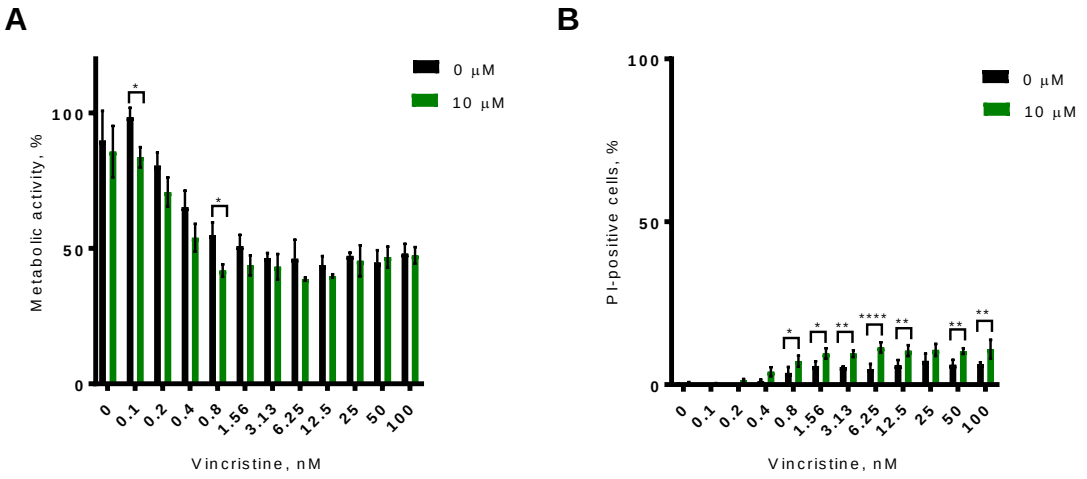

Figure 5.11: Effect of SB203580 and vincristine on U937 cell death
U937 cells were pre-treated with 0 or 10 µM SB203580 at 37°C for 1 h to inhibit p38 signalling. Cells were then treated with various concentrations of vincristine. Cells were then incubated at 37°C for 24 h before an (A) MTS assay or (B) PI assay was performed. Data presented as mean ± SD; n=3. Statistical differences shown between SB203580 and no inhibitor at vincristine concentration points, calculated using two-way ANOVA with Sidak's *post hoc* test: * (P<0.05), ** (P<0.01), **** (P<0.0001).

5.4.4 Effect of p38 inhibition and heat treatment

5.4.4.1 Effect of SB202190 and heat treatment on U937 cell death

The effect of p38 inhibition and heat treatment was initially investigated using SB202190. Assessment of metabolic activity after 24 h showed this was significantly ($P<0.0001$) lower for heat treated cells compared to non-heat treated cells, when pre-treated with equivalent concentrations of SB202190 (Figure 5.12A). However, there was a similar difference in metabolic activity between heat treated and non-heat treated cells in the absence of SB202190. A greater difference was observed for 50 µM SB202190.

Results of flow cytometry (Figures 5.12B-D), assessing cell viability 24 h after treatment are consistent with the results observed by the MTS assay. Cell viabilities were significantly ($P<0.01$) lower for heat treated cells compared to non-heat treated cells, in both the presence and absence of SB202190, with the greatest difference being observed for 50 µM SB202190 (Figure 5.12B). SB202190 and heat treatment had minimal effect on early apoptosis (Figure 5.12C), the lower cell viabilities were due to significant ($P<0.01$) increases in the percentages of late apoptotic/secondary necrotic cells (Figure 5.12D).

After 48 h, there were no significant differences in metabolic activities between heat treated and non-heat treated cells pre-treated with 0 to 25 µM SB202190 (Figure 5.12E). For cells pre-treated with 50 µM SB202190, the metabolic activity for heat treated cells was significantly ($P<0.0001$) lower. SB202190 and heat treatment also had minimal effects on necrosis after 48 h (data not shown).

Assessment of cell viability after 48 h using flow cytometry however showed that for cells pre-treated with 12.5 to 50 µM SB202190, cell viabilities were significantly ($P<0.01$) lower for heat treated cells compared to non-heat treated cells (Figure 5.12F). The decrease in cell viabilities for heat treated cells, pre-treated with SB202190, was due to increases in both the percentages of apoptotic (Figure 5.12G) and late apoptotic/secondary necrotic (Figure 5.12H).

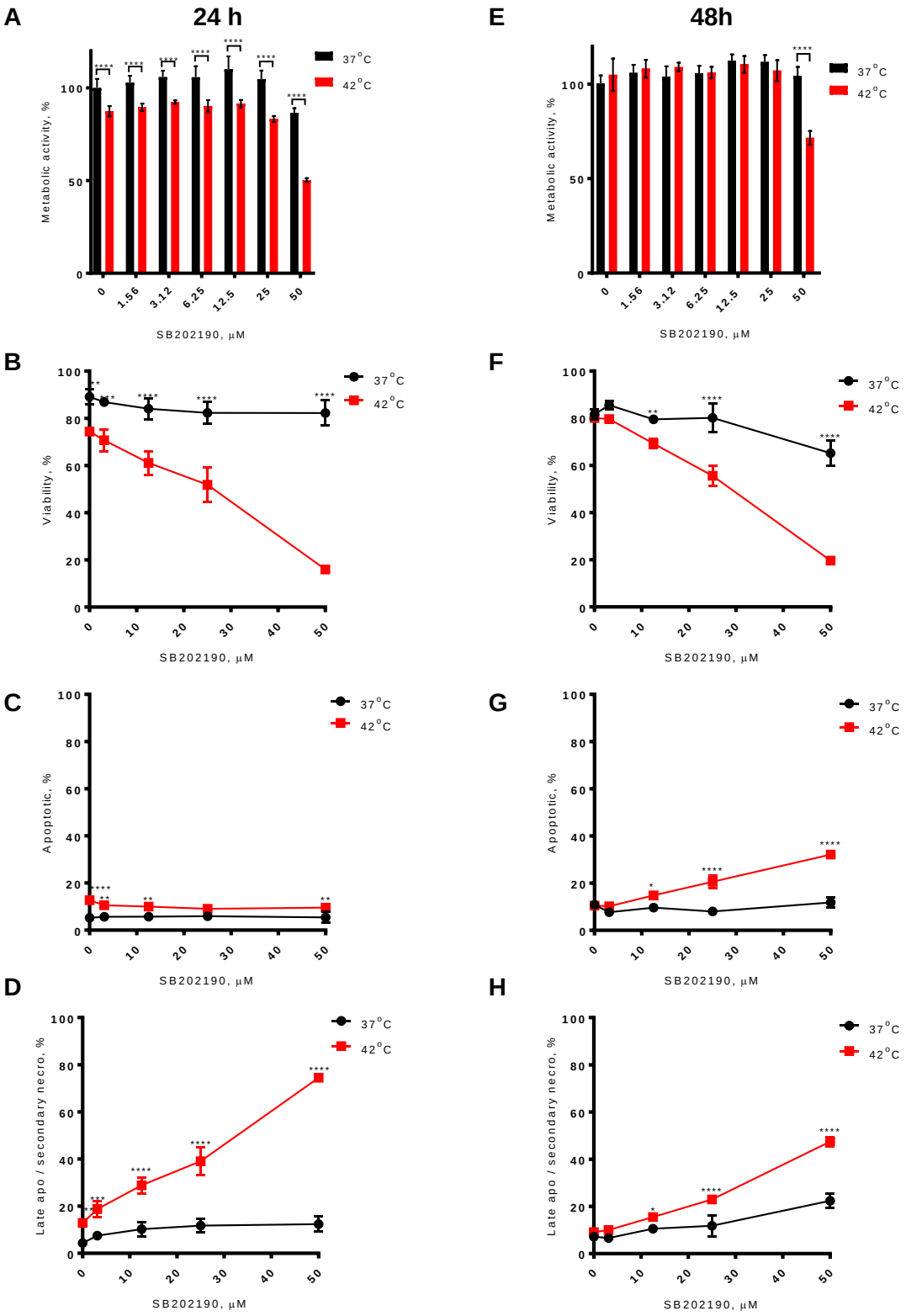

Figure 5.12: Effect of SB202190 & heat treatment on U937 cell death
U937 cells were pre-treated with SB202190 at 37°C for 1 h to inhibit p38 signalling and then incubated at either 37°C or 42°C for 1 h. Cells were then incubated at 37°C for (A-D) 24 h or (E-H) 48h before an (A,E) MTS assay, (B-D,F-H) BD Pharmingen™ FITC Annexin V Apoptosis Detection Kit I were performed. Data presented as mean ± SD; n=3. Statistical differences shown between heat treatment and no treatment at SB202190 concentration points, calculated using two-way ANOVA with Sidak's *post hoc* test: * (P<0.05), ** (P<0.01), *** (P<0.001).

5.3.4.2 Effect of SB203580 and heat treatment on U937 cell death

The role of p38 signalling in the response to heat treatment was further investigated using SB203580. For cells treated with 1.56 to 50 µM SB203580, significant ($P<0.05$) difference in metabolic activities between heat treated and non-heat treated cells were measured after 24 h (Figure 5.13A), however the differences were minimal. SB203580 and heat treatment also had little effect on necrosis after 24 h (data not shown).

Assessment of cell viability after 24 h, using flow cytometry, showed cell viabilities were significantly ($P<0.01$) lower for heat treated cells compared to non-heat treated cells when treated with 12.5 to 50 µM SB203580 (Figure 5.13B). These observed differences were due to significantly ($P<0.05$) higher percentages of apoptotic and late apoptotic/secondary necrotic cells for heat treated cells (Figures 5.13C-D). Cell viability was however also significantly ($P<0.0001$) lower for heat treated cells, compared to non-heat treated cells, in the absence of SB203580 treatment (Figure 5.13B).

After 48 h, inhibiting p38 using SB203580, appeared to have little effect on the difference in metabolic activities between heat treated and non-heat treated cells (Figure 5.13E). Flow cytometry confirmed there were no significant differences in cell viabilities between heat treated and non-heat treated cells for cells pre-treated with 0 to 25 µM SB203580, but the cell viability was significantly ($P<0.001$) lower for heat treated cells pre-treated with 50 µM SB203580 (Figure 5.13F). The observed difference for 50 µM was due to significantly higher percentages of apoptotic ($P<0.0001$) and late apoptotic ($P<0.001$) cells for heat treated compared to non-heat treated cells (Figures 5.13G-H). Taken together, the results suggest inhibition of p38 signalling using SB203580 has minimal effect on heat-induced changes in cell death after both 24 and 48 h.

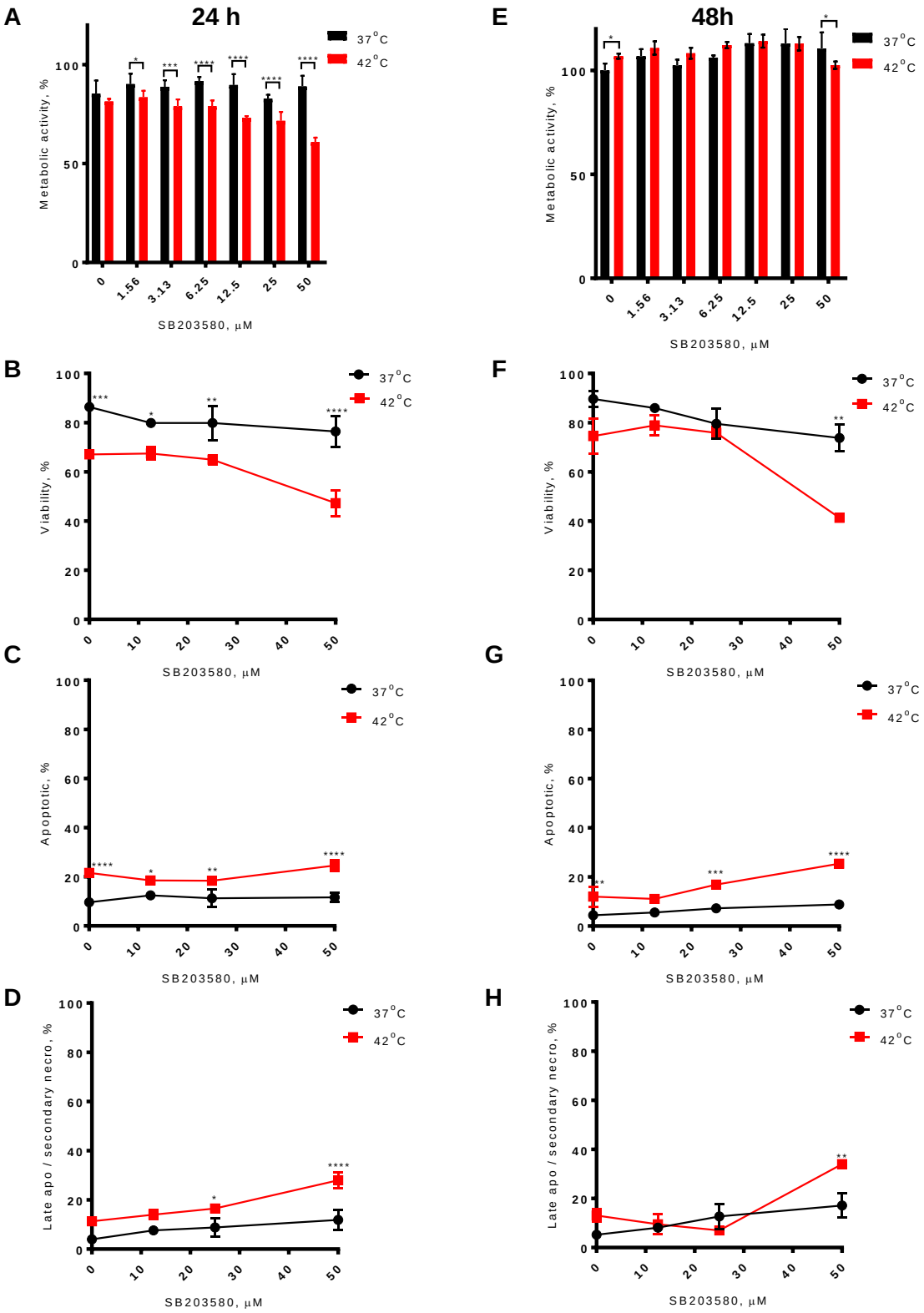

Figure 5.13: Effect of SB23580 and heat treatment on U937 cell death
U937 cells were pre-treated with SB203580 at 37°C for 1 h to inhibit p38 signalling and then incubated at either 37°C or 42°C for 1 h. Cells were then incubated at 37°C for (A-D) 24 h or (E-H) 48h before an (A,E) MTS assay, (B-D,F-H) BD Pharmingen™ FITC Annexin V Apoptosis Detection Kit I were performed. Data presented as mean ± SD; n=3. Statistical differences shown between heat treatment and no treatment at SB203580 concentration points, calculated using two-way ANOVA with Sidak's *post hoc* test: * (P<0.05), ** (P<0.01), *** (P<0.001).

5.4 Discussion

The overall aim of this chapter was to gain a greater understanding of the role of p38 signalling in leukemic cell death by studying the effects of p38 signalling inhibition and cell stressors on cell death in the model U937 cell line.

5.4.1 Inhibition of p38 signalling

The p38 inhibitors, SB202190 and SB203580, were used to target p38 signalling in this study. SB202190 and SB203580 are structurally similar ATP-competitive inhibitors and have both been used extensively in *in vitro* studies to investigate the role of p38. SB202190 binds to p38 kinase in the ATP binding pocket and prevents the phosphorylation of p38 (Fox et al., 1998). SB203580 binds to the ATP binding pocket of p38, and prevents p38 catalytic activity (Young et al., 1997), but does not inhibit the phosphorylation of p38 by upstream kinases (Sanjay Kumar, Jiang, Adams, & Lee, 1999). The aim here was to confirm the inhibition of p38 signalling in U937 cells, using both SB202190 and SB203580, and to establish effective concentrations to use in combination with cell stressors.

5.4.1.1 SB202190

The effect of SB202190 on U937 cell death was assessed and results showed concentrations of SB202190, 25 µM or less, had minimal effects on U937 cell death (Section 5.3.1.1). Western blotting and flow cytometry confirmed p38 phosphorylation was inhibited by 10 µM or higher SB202190 (Section 5.3.1.2). Only partial inhibition of p38 phosphorylation was achieved using 10 µM SB202190, which was consistent with flow cytometry data for 12.5 µM SB202190. SB202190 is selective for p38α and p38β compared to the p38ɣ and p38σ isoforms (Davies, Reddy, Caivano, & Cohen, 2000). Neither of the techniques used in this study to measure p38 phosphorylation were able to establish the effects of SB202190 on the different p38 isoforms as both the antibodies used recognise all isoforms of p38 dually phosphorylated at Thr180 and Tyr182. In order to confirm the ability of SB202190 to specifically inhibit the phosphorylation of p38α and p38β isoforms in U937 cells, antibodies specific for these isoforms would need to be used.

The effect of SB202190 on other MAPKs was not tested in this chapter, as it was believed SB202190 is highly selective for p38α and p38β compared to a range of other, closely related protein kinases (Davies et al., 2000). Whilst kinase assays have shown that SB202190 is selective for p38 inhibition, compared to JNK inhibition (Davies et al., 2000), in cell-based experimental systems, SB202190 induces activation of JNK in A459 lung carcinoma cells and MCF-7 breast cancer cells (Muniyappa & Das, 2008). Although this is the case for A459 and MCF-7 cells, it is possible SB202190 could activate JNK in U937 cells; further testing is necessary to confirm if this is the case.

Previous studies have utilised SB202190 in U937 cells, at concentrations of 10 µM (Liu & Chang, 2010) and 20 µM (Huh, Kang, Chae, & Kim, 2004). Although both studies showed 10 and 20 µM SB202190 had no effect on cell viability, which is consistent with the results presented in this chapter, neither studies confirmed the inhibition of p38 phosphorylation using these concentrations of SB202190. As the results presented here showed that 10 SB202190 was able to inhibit p38 phosphorylation, but had minimal effect on cell viability, 10 µM was selected as an appropriate concentration of SB202190 to pre-treat cells with prior to treatment with cell stressors.

5.4.1.2 SB203580

The effect of an alternative p38 inhibitor, SB2035800, on U937 cell death was assessed and results showed SB203580 concentrations of 25 µM or less had no effect on U937 cell death (Section 5.3.1.3)

Initially the effect of SB203580 on p38 phosphorylation was measured using Western blotting and flow cytometry (Section 5.3.1.4). Flow cytometry showed p38 phosphorylation was inhibited by SB203580 but only very low levels of inhibition were achieved. Western blotting showed SB203580 had no effect on p38 phosphorylation. The minimal effect of SB203580 on p38 phosphorylation was as expected as SB203580 inhibits the catalytic activity of p38 and prevents the phosphorylation of downstream targets, rather than preventing the phosphorylation of p38 itself, by upstream targets (Sanjay Kumar et al., 1999).

Therefore, further confirmation of p38 signalling inhibition in U937 cells, using SB203580, was required. The phosphorylation of MAPKAPK-2 was

selected for investigation as SB203580 has previously been shown to inhibit the activation of MAPKAPK-2 and subsequent phosphorylation of HSP27 (Cuenda et al., 1995). The effect of SB203580 on the phosphorylation of MAPKAPK-2 was measured in this chapter by Western blotting but the Western blot was not clear; further optimisation of phospho-MAPKAPK-2 blot is needed. Without confirmation of the effect of SB203580 on MAPKAPK-2 phosphorylation, there is limited evident to show SB203580 is effective at inhibiting p38 signalling in U937 cells. This therefore limits the conclusions which can be drawn in future sections, where SB203580 is used in combination with cell stressors.

10 µM SB203580 has however been typically used in studies utilising U937 cells (Cuozzo, Raciti, Bertelli, Parente, & Di Renzo, 2012; Hisatsune et al., 2008; Hyun Soo Park et al., 2013; Tauchi et al., 2006; Wang, Zhou, He, & Chen, 2007;Zhang et al., 2012). As the results of this chapter show 10 and 25 µM SB203580 has minimal effect of U937 cell death, and achieves low levels of p38 phosphorylation inhibition, 10 and 25 µM SB203580 were selected for use in combination with cell stressors.

5.4.2 p38 inhibition and UV

The effect of p38 inhibition and UV light on U937 cell death was initially investigated in combination with SB202190 and results showed pre-treatment with SB202190 increased the susceptibility of U937 cells to UV-induced cell death (Section 5.3.2.1). The effect of p38 signalling inhibition and UV on U937 cell death was investigated further using the alternative p38 inhibitor, SB203580 (Section 5.3.2.2). Results showed that although SB203580 increased the susceptibility of U937 cells to UV, the effect was minimal. The effects observed using SB203580 were not as great as those observed using SB202190, but this is likely to be due to different levels of p38 inhibition achieved by the different p38 inhibitors.

Although it is widely believed UV light activates p38 signalling and subsequent apoptosis, p38 has also been implicated in providing protection against UV-induced apoptosis (Chouinard, Valerie, Rouabhia, & Huot, 2002; Gupta, Hoffman, & Liebermann, 2006; Ivanov & Ronai, 2000; Nemoto, Xiang, Huang, & Lin, 1998a). However, there is conflicting evidence for the role of

p38 signalling in UV-induced cell death specifically in U937 cells. Inhibition of p38 signalling, using SB203580, has been reported to have no effect on U937 cell death induced by UV light (Franklin, Srikanth, Kraft, & Prescott, 1998). An alternative study has argued SB203580 increases the level of UV-induced apoptosis (Luchetti et al., 2009); the results presented in this chapter are in agreement with this study, but in addition, the results presented in the chapter confirm the effect using an additional p38 inhibitor, SB202190. In this thesis, cell death was measured by MTS assay and annexin V binding/ PI staining; Luchetti et al (2009) also used annexin V binding/PI staining to measure apoptosis, but in addition confirmed the effects on cell death by measuring total caspase activity and intracellular levels of Bcl-2. The study showed SB203580 and UV light treatment had no effect on the total number of apoptotic cells, compared to UV-only treated cells. SB203580 resulted in a lower percentage of cells in the early stages of apoptosis and a higher percentage of late apoptotic/secondary necrotic cells; the results presented in this chapter are in agreement with these findings. Franklin et al. (1998) used 10 µM SB203580, as was used in this chapter, but cell death was assessed at a different time point (8 h following UV treatment) and using different methods (caspase-3 activity and DNA fragmentation) which could account for the inconsistency in the results. Luchetti et al (2009) also showed the effects of SB203580 and UV light on p38, and additionally ERK phosphorylation. In order to understand the mechanism underlying the observed findings, the effects of SB202190 and SB203580 on MAPK phosphorylation and other proteins needs to be investigated.

5.4.3 p38 inhibition and chemotherapeutic agents

The aim of this section was to investigate the role of p38 signalling in doxorubicin and vincristine-induced apoptosis is U937 cells in order to gain a greater understanding of the mechanisms of chemotherapy resistance in leukaemia.

5.4.3.1 Doxorubicin

The effect of SB202190 and doxorubicin treatment on U937 cell death was assessed and results showed inhibition of p38 signalling, using SB202190 increased the susceptibility of U937 cells to doxorubicin-induced reductions in metabolic activity (Section 5.3.3.1). Measurement of active caspase-3/7 levels however showed pre-treatment with SB202190 had no effect on doxorubicin-induced apoptosis. Further work is required to confirm the effect of SB202190 and doxorubicin on apoptosis.

Cell death following treatment with an alternative p38 inhibitor, SB203580, and doxorubicin was investigated and it was shown SB203580 had no effect on doxorubicin-induced cell death in U97 cells (Section 5.3.3.2). SB203580. However, without confirmation of the effects of 10 µM SB203580 on p38 signalling (as discussed in Section 5.4.1.2), it is not clear whether the lack of observed differences was due to the redundant role of p38 signalling in doxorubicin-induced cell death or due to the inhibitor not effectively inhibiting p38 signalling.

p38 is activated in response to doxorubicin treatment in a range of cancer cell lines such as KB-3 cervical adenocarcinoma cells (Stone & Chambers, 2000) and lung cancer cells (Elsea, Roberts, Druker, & Wood, 2008) and p38 signalling has been associated with resistance to doxorubicin (Chen et al., 2012; Tan, Yu, & Luo, 2014). p38 activation is involved in the resistance of gastric cancer cells to doxorubicin as inhibition of p38 signalling, using SB203580, reduced the viability of doxorubicin-treated gastric cancer cells (Tan et al., 2014). SB203580 and doxorubicin caused an increase in intrinsic (mitochondrial) apoptosis, as evident by an increase in the expression of the pro-apoptotic protein Bax and a decrease in the expression of the anti-apoptotic protein Bcl-2. SB203580 and doxorubicin treatment also suppressed tumour growth *in vivo*. In specific relation to leukaemia, a previous study has implicated the role of p38 signalling in doxorubicin resistance in an alternative leukemic cell line, K562 cells (Chen et al., 2012). Inhibition of p38 signalling, using SB202190, reduced doxorubicin-induced drug resistance in K562 cells which was associated with the decreased expression of P-glycoprotein (PGP) and multidrug resistance protein 1 (MDR1). The results presented in this

chapter using U937 cells, are in agreement with the study conducted by Chen et al. in K562 cells.

5.4.3.2 Vincristine

Cell death following SB202190 and vincristine treatment was assessed, and it was shown that inhibition of p38 signalling, using SB202190, provides protection against vincristine-induced apoptosis in U937 cells. (Section 5.3.3.3). In contrast, inhibition of p38 signalling, using SB203580 had minimal effects on vincristine-induced changes in cell death (Section 5.3.3.4). Given the striking differences observed using SB202190, it was expected that similar results would be observed using SB203580. However, without confirmation of p38 signalling inhibition by 10 µM SB203580, as discussed in Section 5.4.1.2, no firm conclusions can be drawn from the data for SB203580. It is possible, that greater differences in cell death might be observed if higher concentrations of SB203580 were used.

Previous studies have implicated the role of p38 signalling in the resistance of cancer cells to vincristine treatment. Levels of phosphorylated p38 were higher in vincristine-resistant mouse lymphocytic leukaemia L1210 cells, compared to vincristine-sensitive cells and inhibition of p38 signalling, using SB203580, resulted in an increased susceptibility of the mouse lymphocytic leukaemia cells to vincristine (Barančík et al., 2001). It is interesting to note that 30 µM SB203580 was utilised in this study, which is higher than the concentration used in this chapter. This could explain why no differences were observed for 10 µM SB203580 and vincristine treatment, although the different cell types may not be comparable. SB203580 also enhanced the vincristine-induced reductions in cell viabilities in two acute lymphocytic leukemic cell lines: REH and 697 cells (Alsadeq et al., 2015). Although the effect of p38 inhibition and vincristine has not previously been studied specifically in U937 cells, the results of this chapter contradict previous findings in other leukaemia cell lines (Alsadeq et al., 2015; Barančík et al., 2001). However, there is convincing evidence presented in this chapter that p38 signalling inhibition, using SB202190, increases the susceptibility of U937 cells to vincristine. Further work is now required to understand the mechanisms underlying this observation.

5.4.4 p38 inhibition and heat treatment

The role of p38 signalling in the response of U937 cells to heat treatment was investigated using the p38 inhibitor SB202910, in combination with heat treatment (Section 5.3.4.1). It was shown that inhibition of p38 signalling, using SB202190 resulted in the U937 cells being more susceptible to heat treatment after 24 and 48 h. Inhibition of p38 signalling using SB203580 prior to heat treatment had minimal effects on heat treatment-induced cell death after 24 and 48 h (Section 5.3.4.2).

Heat treatment has previously been shown to activate p38 signalling in non-leukemic cells (Dorion, Bérubé, Huot, & Landry, 1999; Dorion, Lambert, & Landry, 2002; Rouse et al., 1994). Although the effect of heat treatment on p38 phosphorylation was not measured in this chapter, it has previously been shown that p38 is activated in U937 cells within 15 min of heat (42°C) treatment, and the heat treatment-induced activation of p38 is inhibited by pre-treatment with 20 µM SB203580 for 30 min (Zhou, An, Xu, Liu, & Cao, 2005).

Heat shock proteins protect cells against stress induced by heat by mediating inhibition of apoptosis and therefore promoting cell survival (Beere, 2004). However it is not established if the activation of p38 following heat treatment is essential for survival. The observation that inhibition of p38 signalling, using SB202190 makes the cells susceptible to heat treatment, suggests a potential involvement of p38 in maintaining cell survival in U937 cells in response to heat treatment. Inhibition of p38 signalling using SB203580 however had minimal effects on heat treatment-induced cell death. The different effects observed using SB20190 and SB203580 could be due to the different mechanisms of inhibition. SB202190 prevents the phosphorylation of p38 (Fox et al., 1998) whereas SB203580 inhibits the catalytic activity of p38 (Young et al., 1997) but does not inhibit the phosphorylation of p38 (Kumar, Jiang, Adams, & Lee, 1999). This suggests that perhaps the phosphorylation of p38 is essential for mediating survival in response to heat treatment. It is surprising SB203580 did not have an effect in the present study as SB203580 inhibits the activation of MAPKAPK-2 and subsequent phosphorylation of heat shock protein 27 (HSP27/HSPB1) (Cuenda et al., 1995). It is possible HSP27 is activated by alternative pathways

or other heat shock proteins play a more prominent role in mediating survival in U937 cells.

5.5 Summary

The results presented in this chapter conclude that SB202190 is effective at inhibiting p38 signalling by preventing the phosphorylation of p38. Although there is some evidence that SB203580 inhibits p38 signalling, by causing minimal reductions in p38 phosphorylation, further work is needed to confirm the ability of SB203580 to inhibit p38 signalling, in particular the effect of SB203580 on MAPKAPK-2 phosphorylation. The lack of effects observed using SB203580 in combination with cell stressors, compared to the effects observed using SB202190 and cell stressors could be due the limited ability of SB203580 to inhibit p38 signalling.

Whilst is has been established that p38 is essential for the survival of ALL and CLL cells (Bendall et al., 2005; Gaundar et al., 2009; Juarez et al., 2009; Ringshausen et al., 2004), there is limited evidence for the role of p38 signalling in the survival of AML cells. The results presented in this chapter show that p38 signalling is important for mediating the survival of U937 cells, an AML cell line, in response to UV light, doxorubicin and heat treatment. Inhibition of p38 signalling, caused an increase in cell death induced by these cell stressors, compared to the stressors alone. In contrast, inhibition of p38 prevented vincristine-induce cell death, suggesting p38 activation is essential for cell death induced by vincristine. The conflicting role of p38 in the survival of U937 cells to two different chemotherapeutic agents highlights the importance of understanding the role of p38 in response to specific chemotherapeutic agents, in order to provide effective therapeutic targeting of p38 signalling.

Chapter 6

Targeting ERK signalling in U937 cell line

Chapter 6: Targeting ERK signalling in U937 cell line

6.1 Introduction

In the previous two chapters, the JNK and p38 signalling pathways have been targeted, which are both pathways activated by stress. In this chapter, ERK signalling is targeted, which in contrast to JNK and p38 signalling, does not have as prominent a role in inducing cell death in response to stress, but rather has an important role in cell growth and survival.

The constitutive activation of ERK signalling has been reported in both leukemic cell lines and primary leukemic cells (Kim et al., 1999; Milella et al., 2001; Morgan et al., 2001; Ricciardi et al., 2005; Towatri et al., 1997). Constitutive ERK activation enables survival of immature or abnormal leukemic cells. Numerous studies have shown inhibition of ERK signalling, using MEK inhibitors, reduces the survival and induces apoptosis in leukemic cells (Kerr et al., 2003; Lunghi et al., 2003; Milella et al., 2001; Morgan et al., 2001). Targeting ERK signalling alone is therefore a promising therapeutic strategy for leukaemia.

Targeting ERK signalling in combination with current chemotherapeutic agents could also potentially increase the efficacy of current leukaemia therapies. A limited number of studies have taken this approach; all-*trans*-retinoic acid, cytarabine and ABT-737 cause cell death in AML cells and inhibition of ERK signalling makes the cells more susceptible to cell death induced by these chemotherapeutic agents (Konopleva et al., 2006; Milella et al., 2001). It is therefore possible that high levels of activated ERK may confer a level of resistance to therapies.

It appears that there are a very limited number of such studies, targeting ERK signalling specifically in U937 cells. This could be due to previous studies stating that untreated U937 cells express very low levels or undetectable levels of phosphorylated ERK (Ajenjo et al., 2000; Milella et al., 2001; Vrana & Grant, 2001). The results presented in Chapter 3 however showed high levels of phosphorylated ERK in U937 cells. Further investigations into the role of ERK signalling in U937 cells are therefore required. In particular to understand if the high levels of phosphorylated ERK observed in U937 cells mediates cell

survival in response to cell stressors, of which UV light and heat treatment will be the focus in this chapter.

Aims

The overall aim of this chapter is to gain a greater understanding of the role of ERK signalling in leukemic cell death using the model U937 cell. This will be achieved by:

- targeting ERK signalling using the chemical inhibitors PD184352 and U0126;
- determining the effects of ERK signalling inhibition on the susceptibility of U937 cells to UV light and heat treatment.

Initially in this chapter, effective concentrations of the ERK inhibitors, PD184352 and U0126, will be established. The effect of the inhibitors on cell death will be investigated using MTS assays, PI assays and flow cytometry to measure annexin V binding and PI staining. The effect of PD184352 and U0126 on ERK phosphorylation will then be assessed by flow cytometry. Due to time restrictions, Western blotting will not be performed. In contrast to previous chapters, the inhibitor will not be used in combination of anisomycin, the effect of each ERK inhibitor alone will be tested. This is based on the results presented in chapter 3 showing ERK phosphorylation is high in untreated U937 cells and is inhibited by anisomycin. The inhibitors will be then used in combination with the cell stressors, which in this chapter will be restricted to UV light and heat treatment. The effect of ERK inhibition and cell stressors on U937 cells will be investigated by using several techniques to assess cell death.

6.2 Methods

6.2.1 Cell culturing

U937 cell line was cultured and maintained as described in Section 2.1.1. Prior to treatments, cells were prepared as described in Section 2.1.4.

6.2.2 Treatment of U937 cells with ERK inhibitors

The ERK inhibitors, PD184352 and U0126, prepared as described in Section 2.2.1, were used in this chapter. U937 cells were treated with PD184352 and U0126 as described in Section 2.2.1.

6.2.3 Measurement of the effects of ERK inhibition on U937 cell death

The effect of ERK inhibition on U937 cell death was assessed using:

- MTS assay to measure metabolic activity (Section 2.4.1.3)
- PI assay to measure necrosis (Section 2.4.1.4)

6.2.4 Measurement of the effects of ERK inhibition on MAPK activation

The effect of ERK inhibition on the phosphorylation of ERK was measured by

- Western blotting (Section 2.3.1)
- flow cytometry (Section 2.3.2)

6.2.5 Treatment of U937 cells with ERK inhibitors and cell stressors

U937 cells were treated with the ERK inhibitors in combination with UV light and heat treatment, as described in Section 2.2.

6.2.6 Measurement of the effects of ERK inhibition and cell stressors on U937 cell death

The effect of ERK inhibition and cell stressors on U937 cell viability was assessed using:

- MTS assay to measure metabolic activity (Section 2.4.1.3)
- PI assay to measure necrosis (Section 2.4.1.4)
- FITC Annexin V FITC Apoptosis Detection Kit I to measure cell viability (Section 2.4.2.1)

6.3 Results

6.3.1 Inhibition of ERK signalling

6.3.1.1 Effect of PD184352 on U937 cell death

Initially the dose-dependent effect of PD184352 on U937 cell death was investigated. As the concentration of PD184352 increased, metabolic activity was reduced in a concentration-dependent manner (Figure 6.1A). Treatment with 0.78 to 6.25 µM PD184352 caused significant ($P<0.05$) decreases in metabolic activities compared to untreated cells; these differences were however minimal. More striking, significant ($P<0.0001$) reductions in metabolic activities were observed for 12.5 µM or higher of PD184352. To establish if the differences were due to cell death by necrosis, a PI assay was performed. Treatment with 0.2 to 6.25 µM PD184352 had no significant effect on necrosis in U937 cells compared to untreated cells (Figure 6.1B). Treatment with 12.5 to 50 µM PD184352 caused significant ($P<0.001$) increases in the percentage of necrotic cells compared to untreated cells. Treatment with 50 µM PD184352, abolished metabolic activity but less than 10% of cells appeared necrotic (Figure 6.1B), which suggests PD184352 induces cell death by a mechanism other than necrosis.

The effect of PD184352 on U937 cell death was investigated further by measuring annexin V binding and PI staining on the flow cytometer. Treatment with 2.5 and 5 µM PD184352 caused a significant ($P<0.01$), but minimal reductions in U937 viabilities (Figure 6.1C). Treatment with 10 µM PD184352 caused a significant ($P<0.0001$) decrease in cell viability (Figure 6.1C) which is consistent with the data for MTS assay (Figure 6.1A). Following treatment with 10 µM PD184352, there was a significant ($P<0.0001$) increase in the percentages of apoptotic (Figure 6.1D) and late apoptotic/secondary necrotic cells (Figure 6.1E). 10 µM PD184352 had no effect on necrosis (data not shown) which suggests the increase observed in the percentage of PI-positive cells (Figure 6.1B) was likely to be due to late apoptotic/secondary necrotic cells (Figure 6.1E). Collectively these results show that whilst concentrations of PD184352 up to 5 µM have minimal effect on cell death, higher concentrations cause cell death.

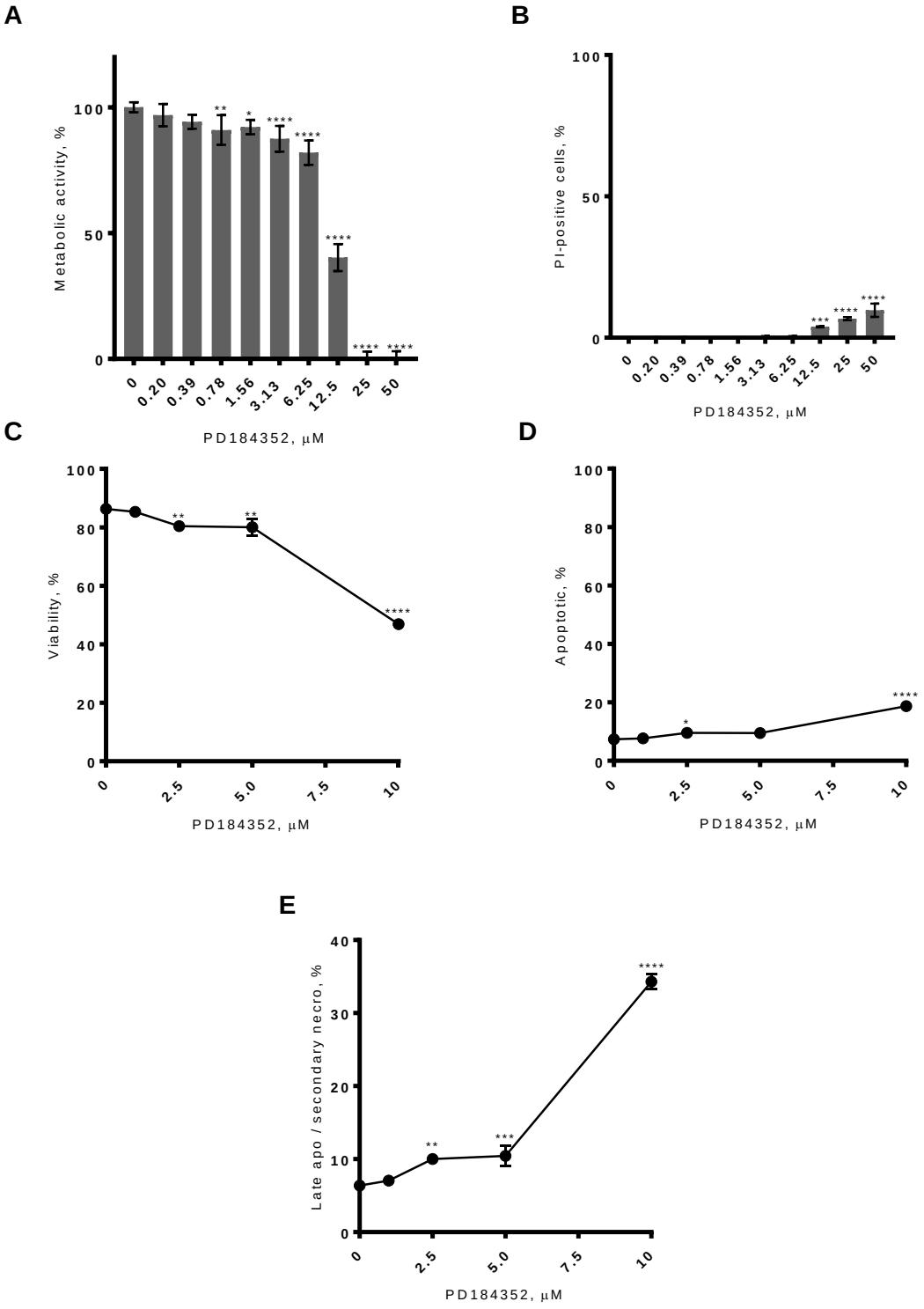

Figure 6.1: Effect of PD184352 on U937 cell death
U937 cells were treated with various concentrations of PD184352 and then incubated at 37°C for 24 h before an (A) MTS assay, (B) PI assay or (C-E) BD Pharmingen™ FITC Annexin V Apoptosis Detection Kit I were performed. Data presented as mean ± SD; n=3. Statistical differences shown to untreated cells, calculated using one-way ANOVA with Dunnett's *post hoc* test: * (P<0.05), ** (P<0.01), *** (P<0.001), **** (P<0.0001).

6.3.1.2 Effect of PD184352 on ERK phosphorylation

The effect of PD184352 on ERK phosphorylation was measured by flow cytometry. Treatment with 5 and 12.5 µM PD184352 caused no significant changes in the percentage of cells expressing phosphorylated ERK compared to untreated cells (Figure 6.2A). The percentages of cells expressing phosphorylated ERK were significantly ($P<0.001$) reduced following treatment with 25 and 50 µM PD184352. Measurement of phospho-ERK mean fluorescence intensity (MFI) showed phospho-ERK MFI was significantly ($P<0.0001$) decreased by over 2-fold for cells treated with 5 to 50 µM PD184352 compared to untreated cells (Figure 6.2B). These results therefore show ERK signalling is inhibited by concentrations of PD184352 as low as 5 µM.

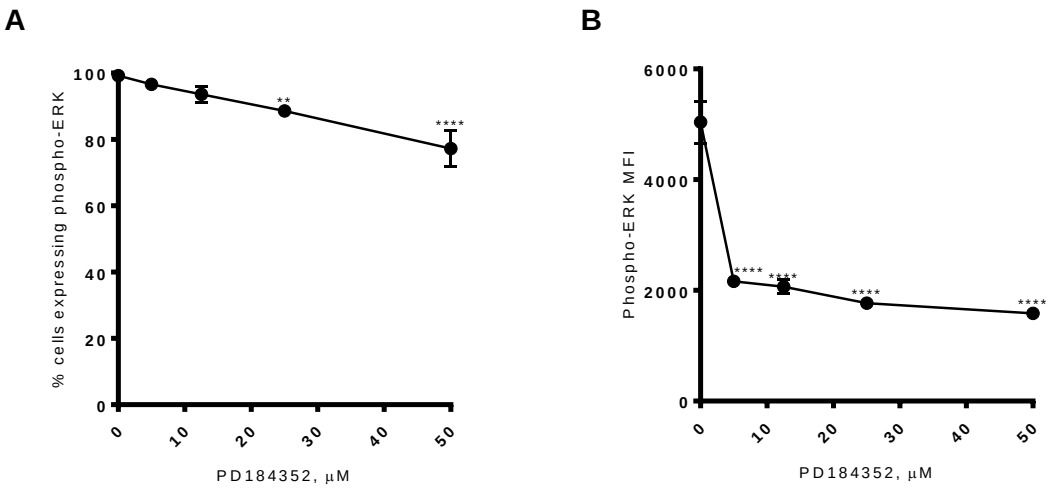

Figure 6.2: Effect of PD184352 on ERK phosphorylation
U937 cells were pre-treated with various concentrations of PD184352 at 37°C for 1 h. Cells were then prepared for flow cytometry to measure ERK phosphorylation. Cells were fixed with 4% paraformaldehyde, permeabilised with 90% ice-cold methanol and stained with phospho-p44/42 (ERK1/2) Alexa Fluor 647-conjugated antibody. (A) shows the percentage of cells expressing phospho-ERK1/2 and (B) shows phospho-ERK1/2 AlexaFluor 647 mean fluorescence intensity (MFI). Data presented as mean ± SD, n=3. Statistical differences shown to untreated cells, calculated using one-way ANOVA with Dunnett's *post hoc* test: ** (P<0.01), **** (P<0.0001).

6.3.1.3 Effect of U0126 on U937 cell death

The effect of an alternative inhibitor, U0126, on U937 cell death was investigated. Results showed metabolic activity was reduced in a concentration-dependent manner following treatment with U0126 (Figure 6.3A). For cells treated with 100 µM U0126, metabolic activity was reduced ($P<0.0001$) to 11%. The effect of U0126 on cell death was further tested by performing a PI assay. Treatment with 1.56 to 6.25 µM U0126 had no significant effect on the percentage of necrotic cells compared to untreated cells (Figure 6.3B). The percentages of necrotic cells were significantly ($P<0.001$) increased in a concentration-dependent manner following treatment with 12.5 to 100 µM U0126.

Flow cytometry analysis of cell viability showed treatment with 5 and 10 µM U0126 did not cause a reduction in U937 cell viability (Figure 6.3C), and consequently had no effect on apoptosis (Figure 6.3D). Treatment with 25 µM U0126 caused a significant ($P<0.001$) decrease in cell viability compared to untreated cells but only by 3% (Figure 6.3C) which was due to an increase in the percentage of late apoptotic/secondary necrotic cells (Figure 6.3D). Taken together, these results show 25 µM or less PD184352 has minimal effects on U937 cell death.

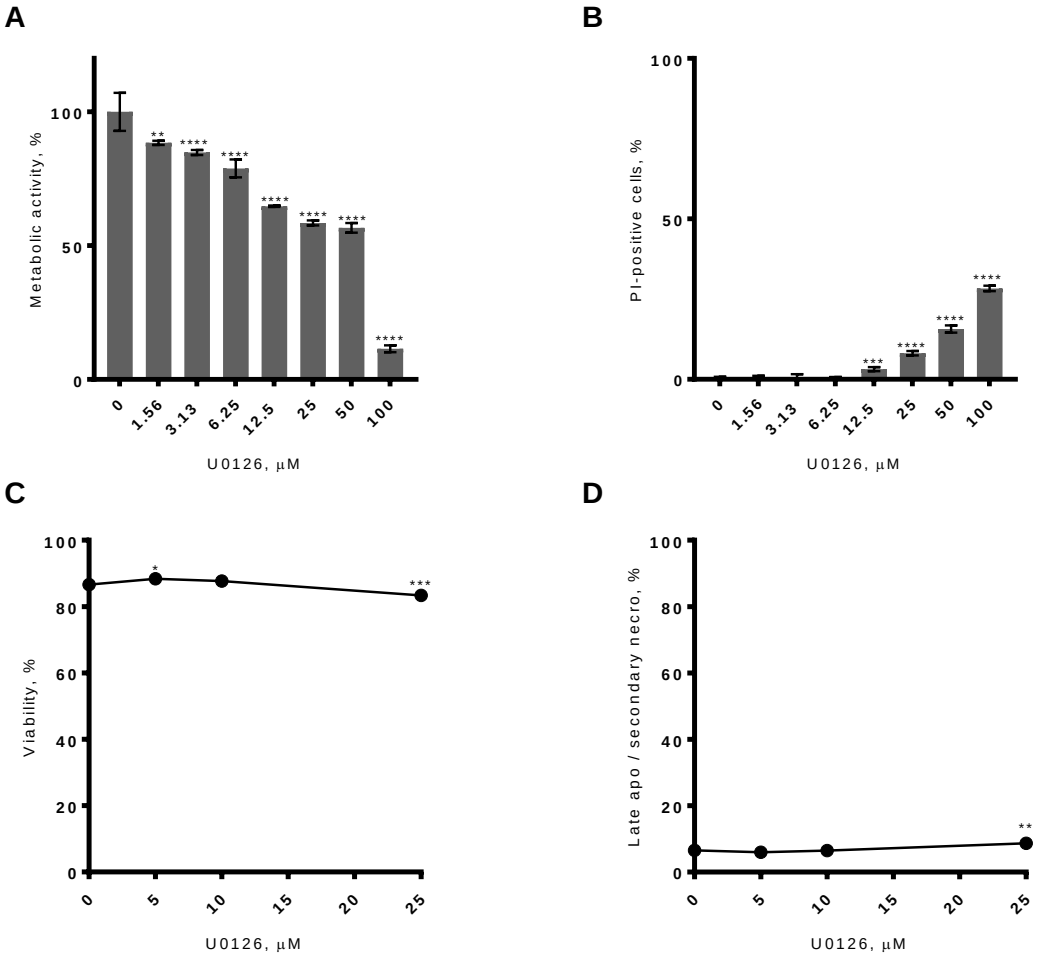

Figure 6.3: Effect of U0126 on U937 cell death
U937 cells were treated with various concentrations of U0126 and then incubated at 37°C for 24 h before an (A) MTS assay, (B) PI assay or (C,D) BD Pharmingen™ FITC Annexin V Apoptosis Detection Kit I were performed. Data presented as mean ± SD; n=3. Statistical differences shown to untreated cells, calculated using one-way ANOVA with Dunnett's *post hoc* test: * (P<0.05), ** (P<0.01), *** (P<0.001), **** (P<0.0001).

6.3.1.4 Effect of U0126 on MAPK phosphorylation

The effect of U0126 on ERK phosphorylation was measured by flow cytometry. Treatment with 5 and 12.5 µM U0126 had no significant effect on the percentages of cells expressing phosphorylated ERK compared to untreated cells (Figure 6.4A). The percentage of cells expressing phosphorylated ERK was significantly (P<0.001) reduced following treatment with 25 µM U0126. Measurement of phospho-ERK mean fluorescence intensity (MFI) showed treatment with 5 µM U0126 caused a significant (P<0.0001) decrease in phospho-ERK MFI compared to untreated cells, by around 2-fold (Figure 6.4B). Phospho-ERK MFIs were decreased further following treatment with 12.5 to 50 µM U0126. Collectively, these results suggest concentrations of U0126 as low as 5 µM can inhibit ERK signalling.

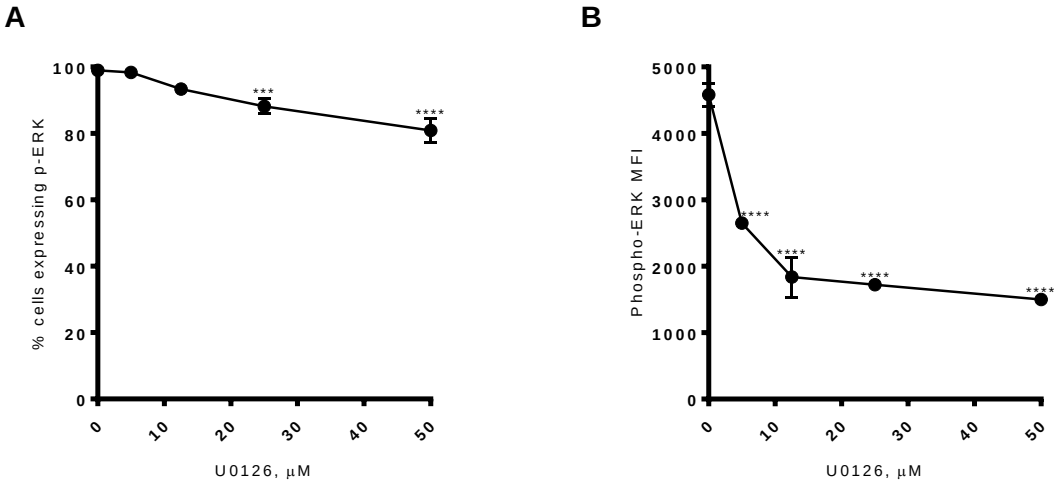

Figure 6.4: Effect of U0126 on ERK phosphorylation
U937 cells were pre-treated with various concentrations of U0126 at 37°C for 1 h. Cells were then prepared for flow cytometry to measure ERK phosphorylation. Cells were fixed with 4% paraformaldehyde, permeabilised with 90% ice-cold methanol and stained with phospho-p44/42 (ERK1/2) Alexa Fluor 647-conjugated antibody. (A) shows the percentage of cells expressing phospho-ERK1/2 and (B) shows phospho-ERK1/2 AlexaFluor 647 mean fluorescence intensity (MFI). Data presented as mean ± SD, n=3. Statistical differences shown to untreated cells, calculated using one-way ANOVA with Dunnett's *post hoc* test: *** (P<0.001), **** (P<0.0001).

6.3.2 Effect of ERK inhibition and UV light

6.3.2.1 Effect of PD184352 and UV light on U937 cell death

The role of ERK signalling in UV-induced cell death was investigated by pre-treating U937 cells with 5 µM PD184352 prior to UV light treatment. For cells treated with 5 to 20s UV, metabolic activities were significantly ($P<0.01$) lower for cells pre-treated with 5 µM PD184352 compared to cells not treated with PD184352 (Figure 6.5A). However, similar differences were observed in the absence of UV. Results of the PI assay showed there were very minimal differences between UV-treated cells pre-treated with PD184352 and cells not pre-treated with PD184352 (Figure 6.5B). Treatment with 5 µM PD184352 therefore has little effect on UV-induced cell death.

A higher concentration of PD184352 was tested. For all durations of UV light, metabolic activities were significantly ($P<0.0001$) lower for cells pre-treated with 10 µM PD184352 compared to cells not treated with PD184352 when treated with equivalent durations of UV light (Figure 6.5D). Similar differences were also observed in the absence of UV light, which is consistent with the data shown in Figure 6.1A. Results of the PI assay showed that for cells treated with 30 to 50 s UV, the percentages of PI-positive cells were significantly ($P<0.05$) higher for cells pre-treated with 10 µM PD184352 compared to cells not treated with PD184352 however the differences were minimal. Taken together, these results show inhibition of ERK signalling using PD184352 has minimal effects on UV-induced U937 cell death.

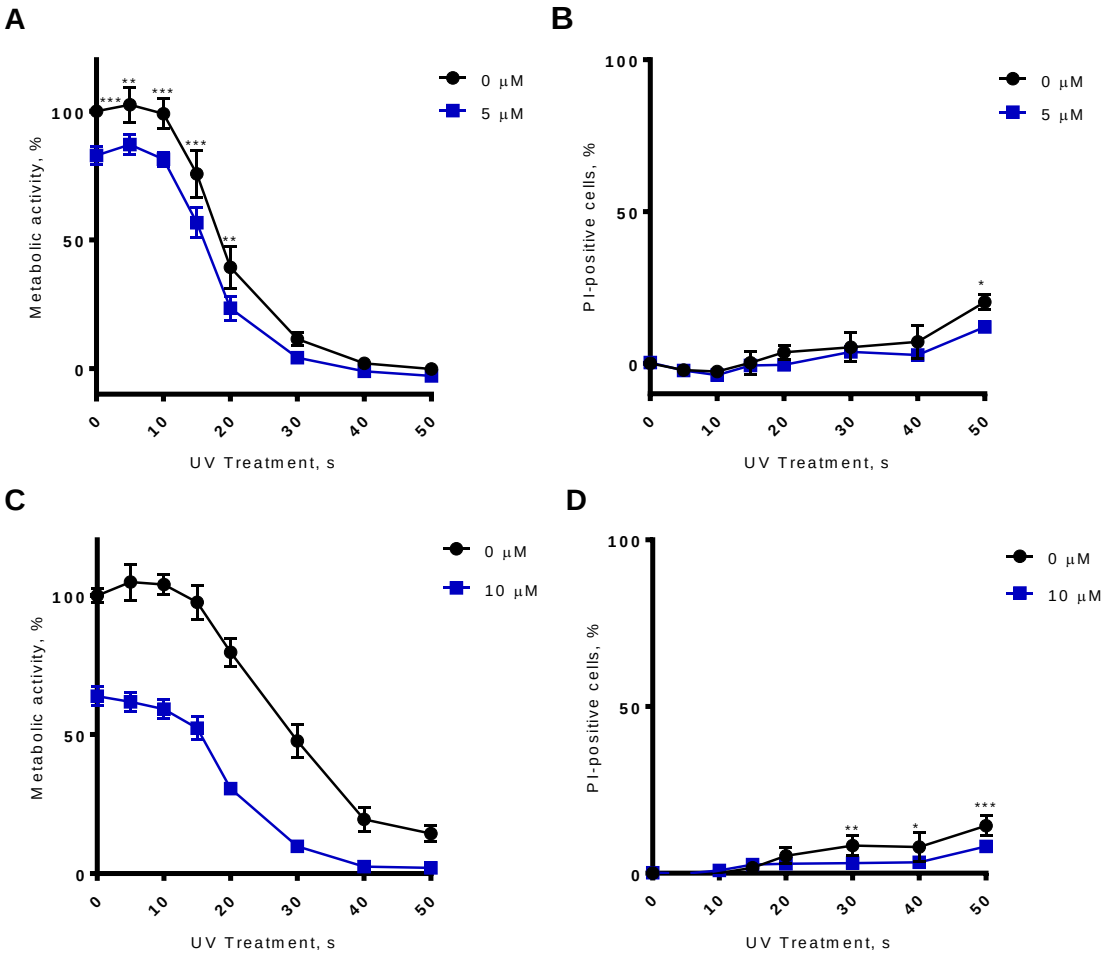

Figure 6.5: Effect of PD184352 and UV light treatment on U937 cell death
U937 cells were pre-treated with (A,B) 5 µM or (C,D) 10 µM PD184352 at 37°C for 1 h to inhibit ERK signalling and then treated with various durations of UV light. After 24 h incubation at 37°C, an (A,C) MTS assay or (B,D) PI assay were performed. Data presented as mean ± SD; n=3. Statistical differences shown between PD184352 and no treatment at mean time points, calculated using two-way ANOVA with Sidak's *post hoc* test: * (P<0.05), ** (P<0.01), *** (P<0.001).

6.3.2.2 Effect of U0126 and UV light on U937 cell death

To further investigate the role of ERK signalling in the response of U937 cells to UV, cells were pre-treated with U0126 prior to UV light treatment, and the effect on cell death was assessed. In the absence of UV, metabolic activity was significantly ($P<0.05$) lower for cells pre-treated with 5 µM U0126 compared to cells not treated with U0126 (Figure 6.6A), which is consistent with Figure 6.3A. For cells treated with 5 to 50s UV, there were no significant differences in metabolic activities between cells treated with and without 5 µM U0126, when treated with equivalent durations of UV (Figure 6.6A). U0126 had no effect on the percentage of PI-positive cells (Figure 6.6B), suggesting U0126 had no effect on necrosis induced by UV light treatment.

The effect of a higher concentration of U0126 was tested. For cells treated with 5 to 30 s UV, metabolic activities were significantly lower ($P<0.05$) for cells pre-treated with 10 µM U0126 compared to cells not treated with U0126 (Figure 6.6C). However, the differences observed were because of the effects of the inhibitor as a similar difference was observed for 0 s UV. 10 µM U0126 had no effect on necrosis in U937 cells induced by UV treatment (Figure 6.6D). Taken together, these results show inhibition of ERK signalling, using U0126, has no effect on UV-induced cell death in U937 cells.

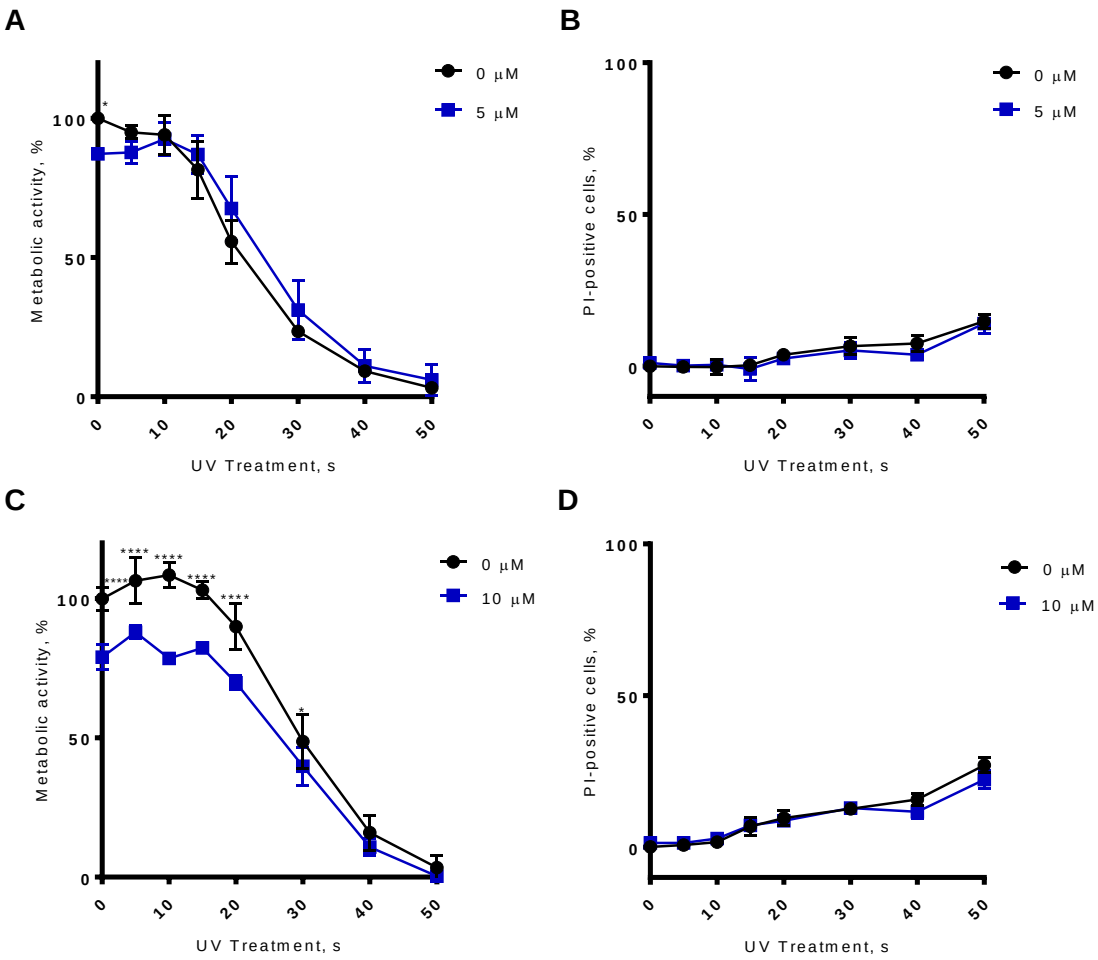

Figure 6.6: Effect of U0126 and UV light treatment on U937cell death
U937 cells were pre-treated with (A,B) 5 µM or (C,D) 10 µM U0126 at 37°C for 1 h to inhibit ERK signalling and then treated with various durations of UV light. After 24 h incubation at 37ºC, an (A,C) MTS assay and (B,D) PI assay were performed. Data presented as mean ± SD; n=3. Statistical differences shown between U0126 and no treatment at mean time points, calculated using two-way ANOVA with Sidak's *post hoc* test: * (P<0.05), **** (P<0.0001).

6.3.3 Effect of ERK inhibition and heat treatment

6.3.3.1 Effect of PD184352 and heat treatment on U937 cell death

The effect of ERK signalling in the response of U937 cells to heat treatment was investigated using PD184352. After 24 h, metabolic activities were significantly ($P<0.0001$) lower for heat treated cells in both the absence and presence of PD184352 (Figure 6.7A). PD184352 had no effect on heat-induced changes in necrosis after 24 h (data not shown). Results from the measurement of cell viability after 24 h using flow cytometry (Figure 6.7B) were consistent with the results of the MTS assay (Figure 6.7A). For cells treated with 0 to 50 µM PD184352, cell viabilities were lower for heat treated cells compared to non-heat treated cells (Figure 6.7B). After 24 h, no significant differences in the percentages of apoptotic cells were measured between heat treated and non-heat treated cells, pre-treated with and without PD184352 (Figure 6.7C) and the significant ($P<0.01$) differences observed for the percentages of late apoptotic/secondary necrotic cells were minimal (Figure 6.7D).

After 48h, no significant differences in metabolic activities were measured between heat treated and non-heat treated cells treated with equivalent concentrations of PD184352 (Figure 6.7E). These results were confirmed by flow cytometry; after 48 h no differences in U937 viability (Figure 6.7F), and hence no differences in the percentages of apoptotic (Figure 6.7G) and late apoptotic/secondary necrotic (Figure 6.7H) cells were measured. Collectively these results show that inhibition of ERK signalling, using PD184352 has no effect on heat-induced changes in cell death.

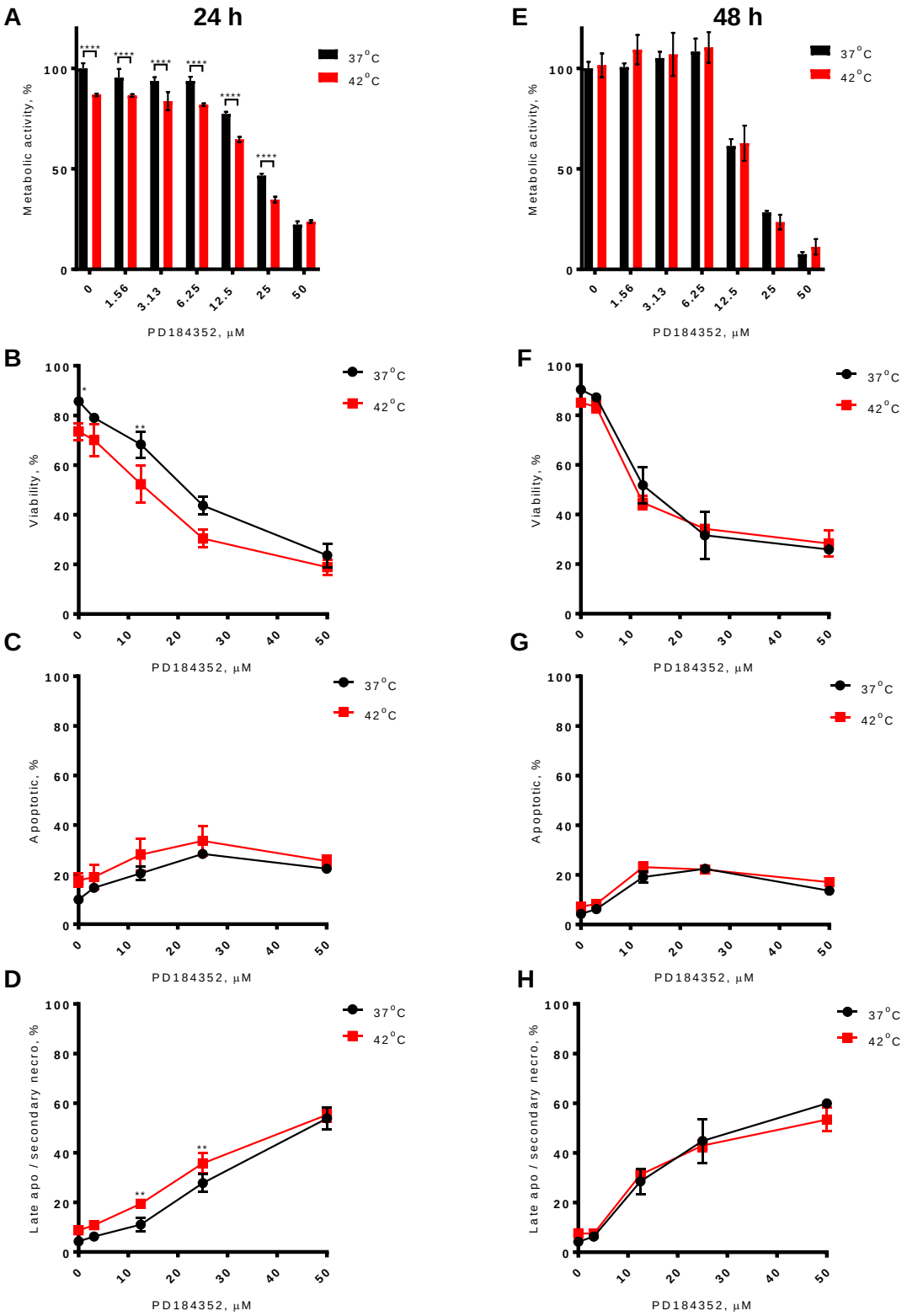

Figure 6.7: Effect of PD184352 and heat treatment on U937 cell death
U937 cells were pre-treated with PD184352 at 37°C for 1 h to inhibit ERK signalling and then incubated at either 37°C or 42°C for 1 h. Cells were then incubated at 37°C for (A-D) 24 h or (E-H) before an (A,E) MTS assay or (B-D,F-H) BD Pharmingen™ FITC Annexin V Apoptosis Detection Kit I were performed. Data presented as mean ± SD; n=3. Statistical differences shown between heat treatment and no treatment at mean PD184352 concentration points, calculated using two-way ANOVA with Sidak's *post hoc* test: ** (P<0.01), **** (P<0.0001).

6.3.3.2 Effect of U0126 and heat treatment on U937 cell death

The effect of U0126 an alternative ERK inhibitor and heat treatment on U937 cell death was initially investigated by performing an MTS assay. After 24 h, metabolic activities were significantly ($P<0.05$) lower for heat treated cells, compared to non-heat treated cells, when treated with 1.56 to 50 µM U0126 (Figure 6.8A). Similar differences were also observed in the absence of U0126. U0126 had no effect on heat-induced changes in necrosis (data not shown). 48 h after treatment, there were no significant differences in metabolic activities between heat treated and non-heat-treated cells, for all concentrations of U0126 (Figure 6.8B). Together, these results suggest inhibition of ERK signalling, using U0126, has no effect on heat-induced changes in U937 cell death and therefore this was not investigated further.

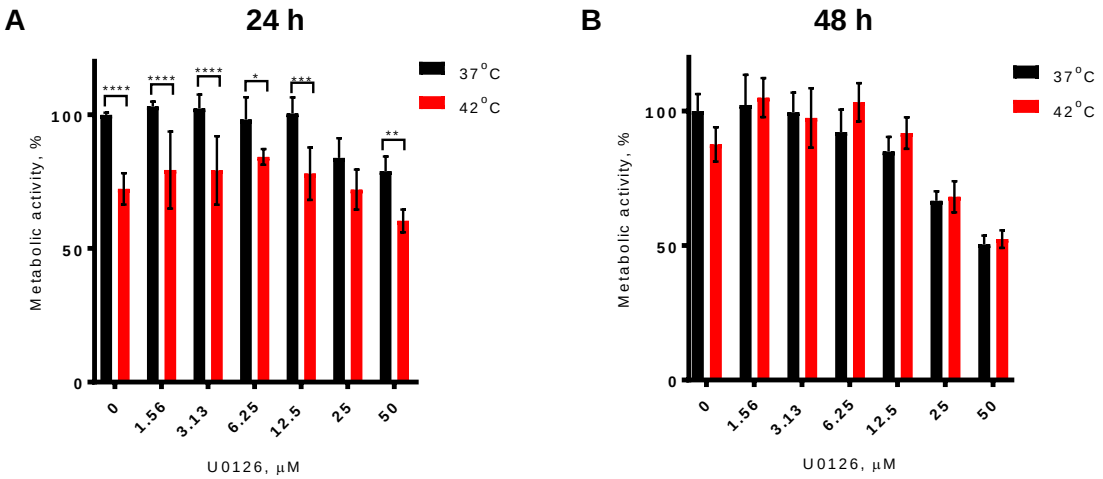

Figure 6.8: Effect of U0126 and heat treatment on U937 cell death
U937 cells were pre-treated with U0126 at 37°C for 1 h to inhibit ERK signalling and then incubated at either 37°C or 42°C for 1 h. Cells were then incubated at 37°C for (A) 24 h or (B) 24 h before an MTS assay was performed. Data presented as mean ± SD; n=3. Statistical differences shown between heat treatment and no treatment at mean U0126 concentration points, calculated using two-way ANOVA with Sidak's *post hoc* test: * (P<0.05), ** (P<0.01), **** (P<0.0001).

6.4 Discussion

The overall aim of this chapter was to gain a greater understanding of the role of ERK signalling in leukemic cell death by studying the effects of ERK inhibition and cell stressors on cell death in the model U937 cell line.

6.4.1 Inhibition of ERK signalling

The ERK inhibitors, PD184352 and U0126, were used in this chapter to target ERK signalling. PD184352 and U0126 are non-competitive inhibitors with respect to ATP and ERK and both function to inhibit MEK1/2 and the subsequent activation of ERK1 and ERK2 (Favata et al., 1998; Sebolt-Leopold et al., 1999). The aim here was to confirm the inhibition of ERK signalling in U937 cells, using PD184352 and U0126, and to establish effective concentrations to use in combination with cell stressors.

6.4.1.1. PD184352

The effect of PD184352 on U937 cell death was assessed and results showed concentrations of PD184352 less than 5 µM, had minimal or no effects on U937 cell death whereas 10 µM or higher of PD184352 did cause low levels of cell death. (Section 6.3.1.1). A previous study utilising PD184352 in U937 cells has shown treatment with up to 10 µM for 24 h has minimal effect on apoptosis (Rahmani et al., 2009). This study is in agreement with the results presented in this chapter and similarly to this chapter, used annexin V staining to assess apoptosis. Other studies have confirmed 10 µM or less PD184352 has minimal effect on apoptosis in U937 cells, using alternative techniques to measure cell death: caspase assays and Wright-Giemsa staining (Dai, Dent, & Grant, 2003; Dai et al., 2001; Yu et al., 2002).

The effect of PD184352 on ERK phosphorylation in U937 cells was measured using flow cytometry (Section 6.3.1.2). Result showed that low concentrations of PD184352 did not affect the percentages of cells expressing phosphorylated ERK, but rather caused the cells expressing phospho-ERK to express phospho-ERK at lower levels. These results therefore provide evidence that concentrations of PD184352 as low as 5 µM are able to partially inhibit ERK signalling.

Measurement of ERK phosphorylation by Western blotting would have provided further confirmation of the effect of PD184352 on the levels of phosphorylated ERK. It has previously been shown ERK phosphorylation, as measured by Western blotting, is inhibited in U937 cells by 10 µM PD184352 after 6 h and remains inhibited after 16 h (Rahmani et al., 2009). Inhibition of ERK phosphorylation has also been observed 18 h after treatment with 10 µM PD184352 (Dai et al., 2001). In this chapter, cells were pre-treated with PD184352 for 1 h prior to treatment with cell stressors, therefore the levels of phosphorylated ERK were measured 1 h after treatment with PD184352, in order to confirm the levels of phosphorylated ERK prior to treatment. In experiments involving co-treatment with PD184352 and a cell stressor, PD184352 was not removed from the culture media; cells were therefore incubated with PD184352 for up to 48 h. It is likely that the lower concentrations of PD814352 would achieve greater inhibition of ERK phosphorylation after time periods longer than 1 h, however this was not tested.

Although high concentrations of PD184352, such as 25 µM, achieved greater inhibition of ERK phosphorylation, these concentrations were not suitable for use due to the high levels of undesired cell death. 10 µM PD184352 has typically been used in previous studies to investigate the role of ERK signalling in U937 cells (Dai et al., 2003; Dai et al., 2001; Rahmani et al., 2009) but the results presented here showed 5 µM is also sufficient to inhibit ERK signalling. 5 µM was therefore selected as an initial concentration of PD184352 to use in combination with cell stressors.

6.4.1.2 U0126

The effect of an alternative ERK inhibitor, U0126, on U937 cell death was tested and results showed significant reductions in metabolic activity and increases in the percentages of necrotic cells following U0126, indicating U0126 has a significant impact on cell death. Flow cytometry, to measure annexin V binding and PI staining, however showed U0126 does not have an effect on apoptosis in U937 cells. The difference in these findings could be attributed to the ability of U0126 to affect cellular processes, other than apoptosis, which may alter metabolic activity such as cell growth and

proliferation. Given the important role of ERK signalling in cell growth and proliferation, this is a plausible explanation. However such effects were not observed for PD184352, which is surprising given that both U0126 and PD18435 inhibit ERK signalling via similar mechanisms. Alternative methods to assess cell growth and proliferation would provide a greater insight into the effect of U0126 on these cellular processes, in addition to further techniques, such as caspase-based assays to confirm the effect of U0126 on apoptosis. The results presented in this chapter however show that concentrations such as 5 and 10 µM U0126 do not have an effect on apoptosis, which is in agreement with a previous study showing treatment with 10 µM U0126 for 24 h has minimal effect on U937 apoptosis (Rahmani et al., 2009).

The effect of U0126 on ERK phosphorylation was measured using flow cytometry (Section 6.3.1.4). Similarly as for PD184352, these result indicate that low concentrations of U0126, such as 5 µM, inhibit ERK phosphorylation, by reducing the levels of phosphorylated ERK expressed by a cell, rather than affecting the number of cells expressing phosphorylated ERK.

Given the minimal effect of U0126 on U937 apoptosis, and the ability of U0126 to inhibit ERK phosphorylation, 5 and 10 µM were selected as appropriate concentrations to use in combination with cell stressors in this chapter. 10 µM U0126 has previously been used to investigate ERK signalling in U937 cells (Su et al., 2014).

6.4.2 ERK inhibition and UV

The effect of ERK inhibition and UV light treatment on U937 cell death was initially investigated by targeting ERK signalling using PD184352 (Section 6.3.2.1). From the results presented, it is evident ERK signalling inhibition, using PD184352 does not have an effect on UV-induced apoptosis in U937 cells. The effects of ERK inhibition and UV light on U937 cell death was investigated further using the alternative ERK inhibitor, U0126 (Section 6.3.2.2). These results also showed U0126 had no effect on UV-induced apoptosis. As the two different ERK inhibitors tested both provide evidence that ERK signalling inhibition does not affect UV-induced cell death, it is evident that ERK signalling does not play a critical role in UV-induced apoptosis in U937 cells.

There is conflicting evidence for the role of ERK in providing cancer cells with protection against UV-induced apoptosis. In C8161 melanoma cells, the activation of ERK, induced by UV light, is inhibited by U0126 but ERK inhibition did not provide protection against UV-induced cell death, as measured by PARP fragmentation (Rieber & Rieber, 2006). ERK activation however has a protective role against UV-induced cell death in A431 epidermoid carcinoma cells (Kitagawa et al., 2002). In U937 cells, the phosphorylation of ERK was decreased following UV and was completely inhibited following PD98059 and UV treatment (Luchetti et al., 2009). However this did not have an effect on UV-induced apoptosis as the percentage of apoptotic cells and caspase activity was unaffected by PD98059 and UV compared to UV-only treated cells. The results in this chapter are in agreement the findings of Luchetti et al (2009).

6.4.3 ERK inhibition and heat treatment

To investigate the effects of ERK inhibition and heat shock on U937 cell death, cells were treated with PD184352 and heat treatment (Section 6.3.3.1). Results showed PD184352 had no effect on U937 cell death 24 or 48 h after heat treatment. The effect of ERK inhibition on heat treatment-induced cell death was investigated further using U0126 (Section 6.3.3.2). U0126 had no effect after 24 nor 48 h, on heat treatment-induced changes in metabolic activities. Collectively these results show ERK signalling is not involved in the response of U937 cells to heat-induced changes in cell death.

ERK signalling has an important role in cell survival and has been implicated in preventing heat-induced cell death. Heat treatment (42°C, 60 min) activated ERK1/2 phosphorylation in BaF3, murine pro-B cells (Ng & Bogoyevitch, 2000). Treatment of BaF3 cells with PD98059, an alternative ERK inhibitor, inhibited the heat treatment-induced activation of ERK and resulted in a greater reduction in viability. ERK signalling, in particular the activation of ERK1 is important for protection against heat-induced cell death in K562, chronic myeloid leukemic cells (Woessmann & Meng, 1999). Inhibition of ERK1 activity increased the sensitivity of K562 cells to heat shock-induced reductions in cell viability, whereas the overexpression of ERK1 resulted in resistance to cell death caused by heat treatment. ERK2 activation

was unaffected by heat treatment (43°C, 20 min) in a range of leukemic cell lines (K562, HL-60 and U937 cells).

The effect of ERK signalling on heat-induced cell death, specifically in U937 cells has not previously been reported. The results of this chapter implicate ERK signalling is not involved in maintaining viability in U937 cells following heat treatment. The finding that ERK2 is not activated by heat treatment in U937 cells (Woessmann & Meng, 1999), provides support for the lack of involvement of ERK signalling observed in this chapter. However Woessmann et al. did not show the effect of heat treatment on ERK1 phosphorylation. It has also been argued that heat treatment (42°C, 15 min or 30 min) does in fact induce activation of ERK in U937 cells and the activation of ERK by heat treatment is inhibited by PD098059 (Zhou et al., 2005). Therefore, in order to fully understand the effects of ERK signalling in the response to heat treatment in U937 cells, the effects of heat treatment on ERK phosphorylation in U937 cells need to be investigated using this experimental system.

6.4 Summary

Whilst it has previously been reported that U937 cells express low levels of phosphorylated ERK (Ajenjo et al., 2000; Milella et al., 2001; Vrana & Grant, 2001), the results presented in this chapter, confirm untreated U937 cells have high levels of phosphorylated ERK, as observed in Chapter 3. PD184352 and U0126 are effective at inhibiting ERK signalling and inhibition of ERK signalling induces apoptosis in U937 cells, consistent with previous findings in other types of leukemic cells (Kerr et al., 2003; Lunghi et al., 2003; Milella et al., 2001). There are limited studies available which demonstrate the role of ERK signalling in mediating U937 cell survival in response to cell stressors, such as UV light and heat treatment. The results presented in this chapter provide evidence that ERK signalling is not essential for the survival of U937 cells in response to UV light and heat treatment but further research is needed to investigate the role in response to other cell stressors, for example chemotherapeutic agents.

Chapter 7

Targeting MAPK signalling in PBMCs

Chapter 7: Targeting MAPK signalling in PBMCs

7.1 Introduction

In previous chapters, the role of MAPK signalling pathways in leukemic cell death has been investigated using U937 cells, a human monocytic cell line. In this chapter, MAPK signalling pathways will be targeted in peripheral blood mononuclear cells (PBMCs) from healthy individuals.

U937 cells are transformed cells which are a powerful *in vitro* research tool for studying leukaemia. Primary cells however are a more suitable model as they more closely mimic the physiology of cells *in vivo* and provide a greater insight into variation between individuals. In order to confirm the effects observed in U937 cells, future work is required to understand the role of MAPK signalling in leukemic death using primary blasts from AML patients. Prior to this, it is necessary to establish if the effects observed in U937 are specific to leukemic cells or whether there are effects on non-leukemic cells; it is important to ensure that unwanted cell death in non-leukemic cells is minimal. Peripheral blood mononuclear cells (PBMCs) are peripheral blood cells with a round nucleus and include lymphocytes and monocytes (Riedhammer, Halbirtter, & Weissert, 2014). PBMCs from healthy individuals are frequently used in leukemic studies; PBMCs from healthy individuals are used as a control to allow comparison between leukemic and non-leukemic cells (Admoni-Elisha et al., 2016; Le Dieu et al., 2009; Morsi et al., 2016).

Constitutive activation of ERK has previously been reported in leukemic cells (Milella et al., 2001; Morgan, Dolp, & Reuter, 2001; Ricciardi et al., 2005) and high levels of active ERK were measured in U937 cells in Chapter 3 (Section 3.3.5) but the levels of phosphorylated ERK need to be measured in PBMCs from healthy individuals to allow for comparisons to primary, non-leukemic cells. The results presented in Chapter 6 showed inhibition of ERK signalling reduced the survival of U937 cells (Section 6.3.1) but ERK signalling was not essential for the survival of U937 cells in response to UV light and heat treatment (Sections 6.3.2 and 6.3.3). As the results presented in this chapter only show preliminary work in PBMCs, ERK signalling will not be targeted in PBMCs, instead JNK and p38 signalling will be the focus. In Chapters 4 and 5, it was shown that inhibition of JNK and p38 signalling, using

SP600125 and SB201290 respectively, sensitised U937 cells to UV-induced apoptosis (Sections 4.3.2.1 and 5.3.3.1). In contrast, inhibition of JNK and p38 signalling provided protection against vincristine-induced apoptosis in U937 cells (Sections 4.3.4.3 and 5.3.5.3). Investigations are required to determine if this effect is specific to leukemic cells or whether PBMCs from healthy individuals are affected in a similar manner.

Aims

The overall aim of this chapter is to gain a greater understanding of the role of MAPK signalling in leukemic cell death using PBMCs from healthy individuals. This will be achieved by:

- Determining the effect of anisomycin and UV light on MAPK activation in PBMCs
- determining the effects of cell stressors, to include anisomycin, UV light and vincristine on PBMC death;
- determining the effects of JNK and p38 signalling inhibition on the susceptibility of PBMCs to UV and vincristine.

Prior to investigating the effects of any treatments on PBMCS, it is necessary to establish if the process of PBMC isolation has any effect on MAPK activation, this will be achieved by measuring the activation of MAPKs using flow cytometry. Following this, the effect of the cell stressors on the activation of MAPKs in PBMCs and the effect on PBMC death will be investigated using flow cytometry. The effects of JNK and p38 inhibition on the susceptibility of PBMCs to UV and vincristine will be investigated using SP600125 and SB202190, respectively, as these inhibitors were shown to be the most effective in chapters 4 and 6. The effect of the inhibitors and cell stressors on PBMC death will only be assessed by flow cytometry to measure annexin V binding and PI staining.

7.2 Methods

7.2.1 PBMC isolation

Whole blood was collected from healthy individuals (Section 2.1.2.1) and PBMCs were isolated using Histopaque®-1077 as described in Section 2.1.2.2.

7.2.2 Preparation of PBMCs prior to experiments

PBMCs, at a concentration of 5 x 10^5 cells/mL were plated into 96-well tissue culture plates under sterile conditions. PBMCs were treated immediately unless otherwise stated. Each experiment was performed on up to 3 individual participants, and in all cases experimental measurements were replicated 3 times.

7.2.3 Treatment of PBMCs with cell stressors

PBMCs were treated with the following cell stressors:

- anisomycin (Section 2.2.5)
- UV light (Section 2.2.2)
- vincristine (Section 2.2.3)

7.2.4 Treatment of PBMCs with MAPK inhibitors and cells stressors

JNK signalling was inhibited in this chapter using SP600125 and p38 signalling was inhibited using SB202190, which were prepared as described in Section 2.2.1. PBMCs were treated with JNK or p38 inhibitors prior to treatment with cell stressors as described in Section 2.2.

7.2.5 Measurement of MAPK phosphorylation

The effects of PBMC isolation, anisomycin treatment and UV light treatment on MAPK phosphorylation in PBMCs were measured by flow cytometry as described in Section 2.3.2.

7.2.6 Measurement of the effects of treatments on PBMC death

The effects of cell stressors alone, or the effects of JNK or p38 inhibition in combination with cell stressors on PBMC viability was assessed using FITC Annexin V FITC Apoptosis Detection Kit I as described in Section 2.4.2.1.

7.3 Results

7.3.1 Effect of PBMC isolation on MAPK phosphorylation

To determine if the process of isolating PBMCs affects MAPK phosphorylation, phosphorylated-JNK, c-Jun, p38 and ERK levels were measured 0, 2 and 24 h after isolation. PBMC isolation had no effect on the percentage of cells expressing phosphorylated JNK after 2 h and although there was a significant ($P<0.05$) increase after 24 h, the difference was minimal (Figure 7.1A). After 2 and 24 h, there were no significant changes in the percentages of PBMCs expressing phosphorylated c-Jun compared to 0 h (Figure 7.1B). Whilst there was no difference in the percentage of cells expressing phosphorylated p38 after 2 h compared to 0 h, there was a significant ($P<0.05$) decrease in the percentage of cells expressing phosphorylated p38 after 24 h, however this difference was minimal (Figure 7.1C). PBMC isolation had no effect on phosphorylated ERK expression 2 h after isolation; there was a significant ($P<0.0001$) increase in phosphorylated ERK 24 h post isolation (Figure 7.1D). Collectively, these results show PBMC isolation does not affect the phosphorylation of JNK, c-Jun or p38. ERK phosphorylation is however affected; ERK signalling was not investigated further in this chapter.

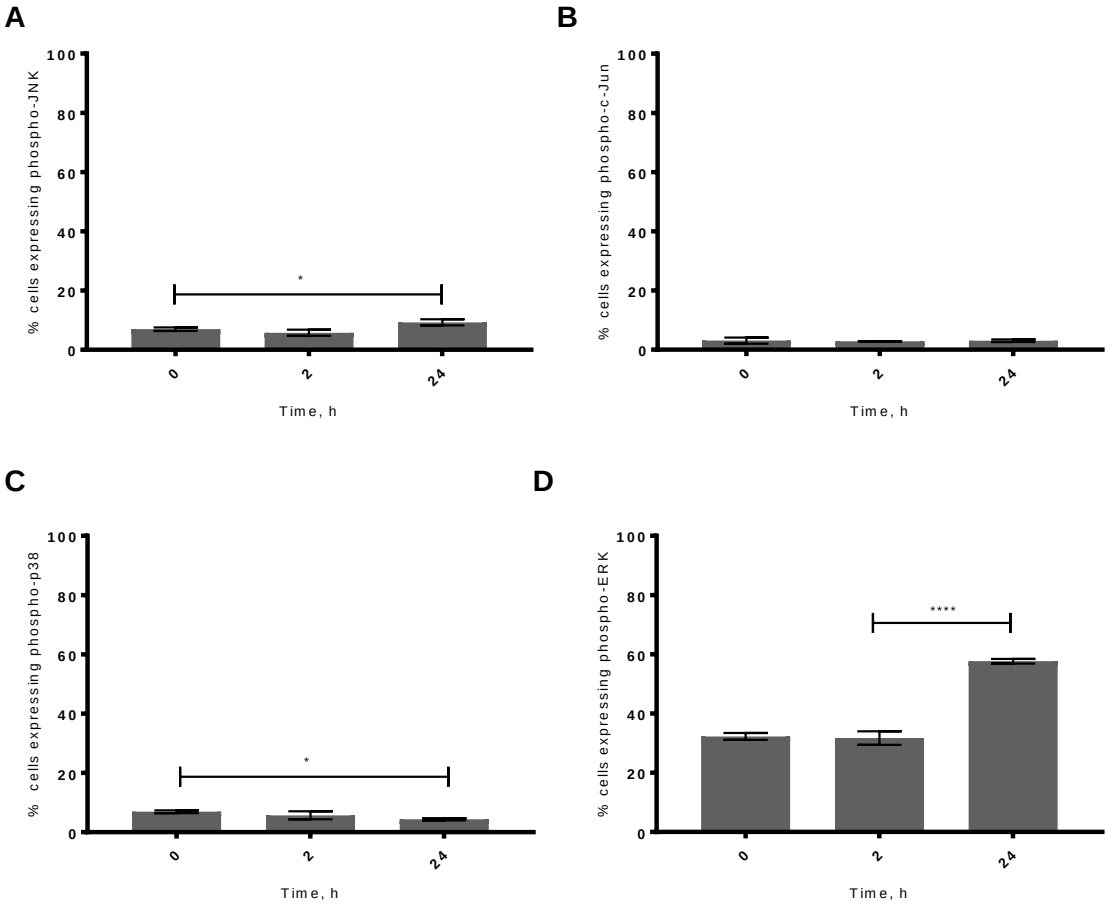

Figure 7.1: Effect of PBMC isolation on MAPK phosphorylation
PBMCs were isolated from healthy human blood using Histopaque-1077 and then incubated at 37°C for 0, 2 or 24 h. PBMCs were then prepared for flow cytometry to measure (A) JNK, (B) c-Jun, (C) p38 and (D) ERK phosphorylation. PBMCs were fixed with 4% paraformaldehyde, permeabilised using 90% ice-cold methanol, and then stained with (A) phospho-SAPK/JNK (Thr183/Thr185) Alexa Fluor 647-conjugated antibody, (B) phospho c-Jun (Ser73) Alexa Fluor 488 conjugated antibody, (C) phospho-p38 (Thr180/Tyr182) Alexa Fluor 647-conjugated antibody or (D) phospho-p44/42 (Thr202/Tyr204) Alexa Fluor 647-conjugated antibody. Data presented as mean ± SD, n=3 experimental replicates for a single participant. Statistical differences shown to 0 h, calculated using one-way ANOVA with Dunnett's *post hoc* test: * (P<0.05), **** (P<0.0001).

7.3.2 Effect of anisomycin on MAPK signalling components in PBMCs

In chapters 3-6 anisomycin was used as a signalling agonist to test the ability of the MAPK inhibitors to inhibit the MAPK signalling pathways. To test if anisomycin could also be used as a signalling agonist in PBMCs, the effect of anisomycin on JNK, c-Jun and p38 phosphorylation was measured by flow cytometry. The percentage of PBMCs expressing phosphorylated JNK was significantly ($P<0.05$) increased 30 min after treatment with 1 µM anisomycin compared to untreated PBMCs; but the difference was less than 3% (Figure 7.2A). The percentages of PBMCs expressing phosphorylated c-Jun and p38 were unaffected by treatment with 1 µM anisomycin for 30 min (Figures 7.2B and 7.2C).

As 1 µM anisomycin had little effect on MAPK phosphorylation in PBMCs, higher concentrations of anisomycin (0 to 50 µM) were tested to ensure that the lack of MAPK activation was not due to insufficient concentration of anisomycin. These investigations were focused on the activation of JNK following anisomycin treatment. There were no significant differences in the percentages of anisomycin-treated PBMCs expressing phosphorylated JNK compared to untreated PBMCs (Figure 7.3).

Longer incubations with a high concentration of anisomycin were also tested. Incubation with 50 µM anisomycin for 30 min had no significant effect on the percentage of PBMCs expressing phosphorylated JNK compared to untreated PBMCs (Figure 7.4A). Treatment with 50 µM anisomycin for 1 h caused a significant ($P<0.01$) increase in the percentage of PBMCs expressing phosphorylated JNK compared to untreated PBMCs but the difference was minimal (Figure 7.4B). Taken together these results suggest anisomycin is unable to activate MAPKs under the conditions tested.

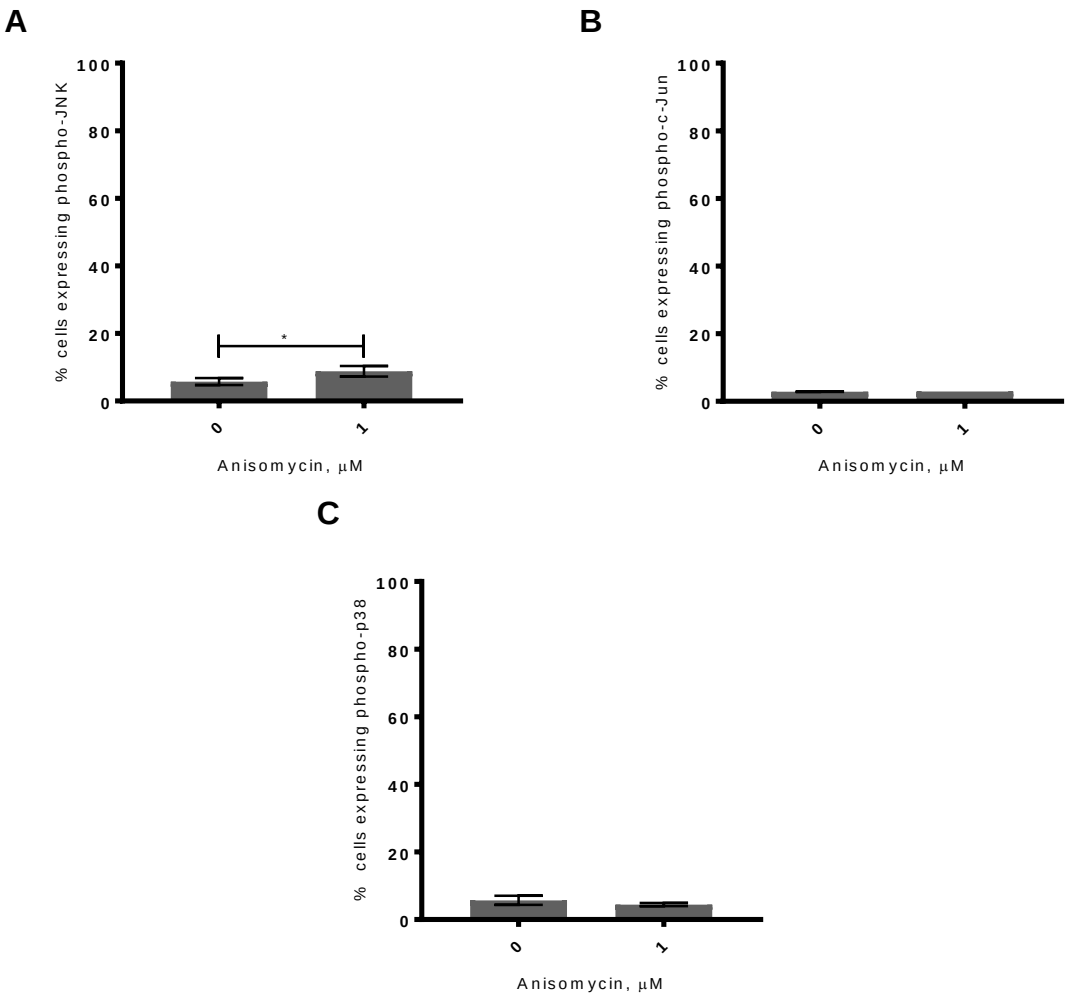

Figure 7.2: Effect of anisomycin on MAPK phosphorylation in PBMCs
PBMCs were incubated with 1 µM anisomycin at 37°C for 30 min and then (A) phospho-JNK, (B) phospho-c-Jun, (C) phospho-p38 levels were then measured using flow cytometry. PBMCs were fixed with 4% paraformaldehyde, permeabilised using 90% ice-cold methanol, and then stained with (A) phoshpo-SAPK/JNK (Thr183/Thr185) Alexa Fluor 647-conjugated antibody, (B) phospho c-Jun (ser73) Alexa Fluor 488 conjugated antibody or (C) phospho-p38 (Thr180/Tyr182) Alexa Fluor 647-conjugated antibody. Data presented as mean ± SD, n=3 experimental measurements for a single participant. Statistical differences shown to untreated PBMCs, calculated using an unpaired t-test: * (P<0.05).

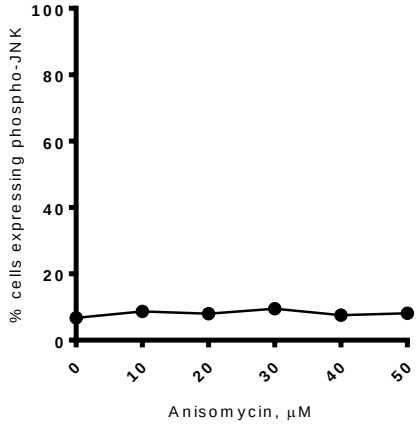

Figure 7.3: Effect of high concentrations of anisomycin on JNK phosphorylation in PBMCs
PBMCs were incubated with 0 to 50µM of anisomycin at 37°C for 30 min and then phospho-JNK, levels were measured using flow cytometry. PBMCs were fixed with 4% paraformaldehyde, permeabilised using 90% ice-cold methanol, and then stained with phospho-SAPK/JNK (Thr183/Thr185) Alexa Fluor 647-conjugated antibody. Data presented as mean ± SD, n=3 experimental measurements for a single participant.

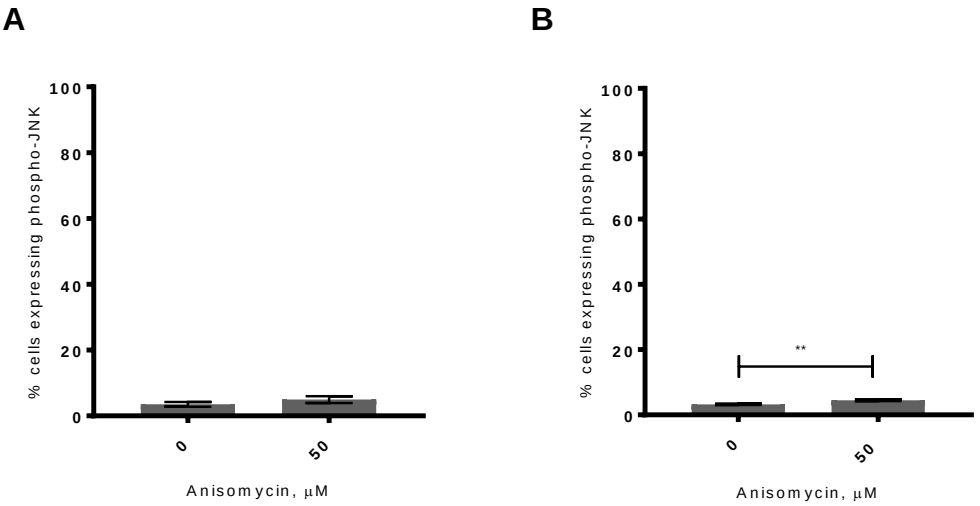

Figure 7.4: Effect of longer incubations with anisomycin on JNK phosphorylation in PBMCs
PBMCs were incubated with 50 µM anisomycin at 37°C for (A) 30 min or (B) 60 min and then phospho-JNK levels were measured using flow cytometry. PBMCs were fixed with 4% paraformaldehyde, permeabilised using 90% ice cold methanol, and then stained with phospho-SAPK/JNK (Thr183/Thr185) Alexa Fluor 647-conjugated antibody. Data presented as mean ± SD, n=3 experimental measurements for a single participant. Statistical differences shown to untreated PBMCs, calculated using an unpaired t-test: ** (P<0.01).

7.3.3 Effect of UV light on PBMC death

To determine the effect of UV light on PBMC death, PBMCs were treated with various durations of UV light and the effect on PBMC cell death was assessed using flow cytometry to measure annexin V binding and PI staining. UV light caused a significant ($P<0.0001$) decrease in PBMC viability (Figure 7.5A). The percentages of necrotic PBMCs were unaffected by UV treatment (data not shown). The decreased viabilities observed in PBMCs following UV treatment were due to increases in both the percentages of apoptotic and late apoptotic/secondary necrotic PBMCs (Figures 7.5B and 7.5C).

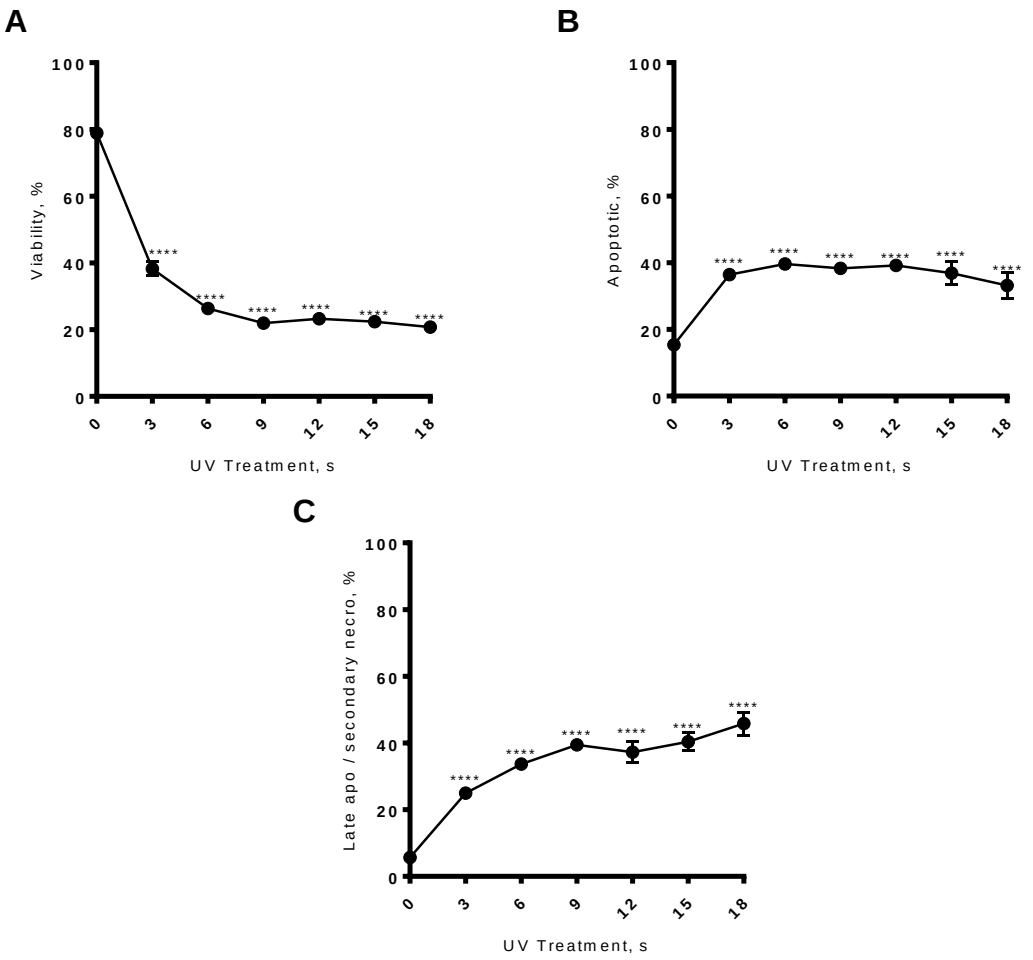

Figure 7.5: Effect of UV light on PBMC viability
PBMCs were treated with various durations of UV light treatment and then incubated at 37°C for 24 h. (A) viability (B) necrosis, (C) apoptosis and (D) late apoptosis/secondary necrosis were measured using BD Pharmingen™ FITC Annexin V Apoptosis Detection Kit I. Data presented as mean ± SD, n=3 experimental replicates for a single participant. Statistical differences shown to untreated PBMCs, calculated using one-way ANOVA with Dunnett's *post hoc* test: **** (P<0.0001).

7.3.4 Effect of UV light on JNK activation in PBMCs

To determine if JNK was activated in PBMCs following UV light treatment, levels of phosphorylated JNK were measured by flow cytometry up to 60 min after UV treatment. Three different UV treatment times were tested: 0, 6 and 18 s. For each duration of UV light, there were no significant changes in the percentages of PBMCs expressing phosphorylated JNK over 60 min, compared to 0 min (Figure 7.6A and Table 7.1). Therefore, the activation of JNK over a longer period of 4 h was studied. There were no significant changes in the percentages of PBMCs expressing phosphorylated JNK after 1, 2, 3, or 4 h compared to 0 h (Figure 7.6B and Table 7.1). These results indicate that JNK signalling is not activated in PBMCs following UV light treatment.

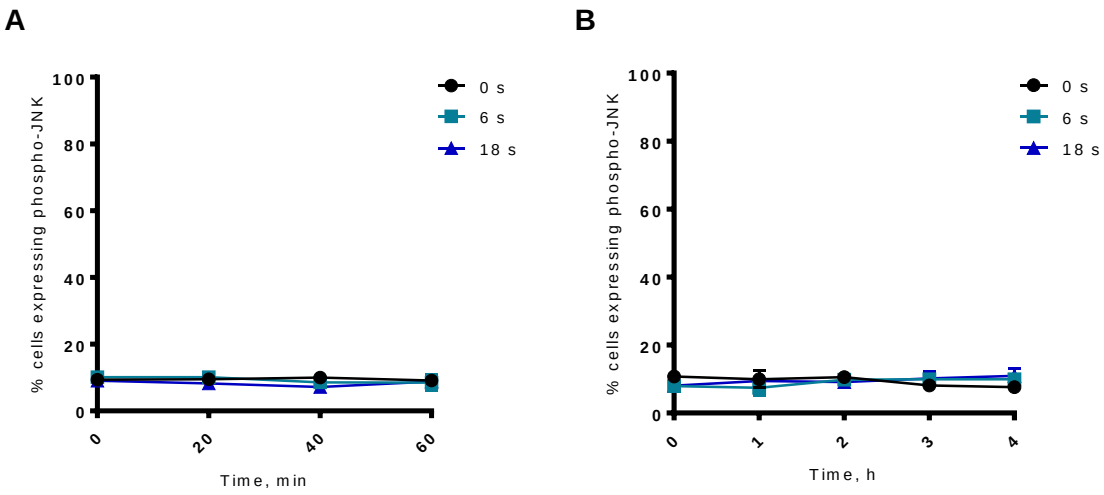

Figure 7.6: Effect of UV light on JNK activation
U937 cells were treated with 0, 6 and 18 s UV, incubated at 37°C for 0 to 60 min and then phospho-JNK levels were then measured using flow cytometry. PBMCs were fixed with 4% paraformaldehyde, permeabilised using 90% ice cold methanol, and then stained with phospho-SAPK/JNK (Thr183/Thr185) Alexa Fluor 647-conjugated antibody. Data presented as mean ± SD, n=3 experimental measurements for a single participant.

Table 7.1: Statistical analysis to show the effect of UV light on JNK activation
Data presented as mean ± SD; n=3 experimental measurements for a single participant. Statistical differences shown between mean incubation times for each UV treatment time, calculated using two-way ANOVA with Tukey's *post hoc* test.

0 – 60 min			
Time, min	**UV Treatment, s**		
	0	**6**	**18**
0 vs 20	ns	ns	ns
0 vs 40	ns	ns	ns
0 vs 60	ns	ns	ns
1 – 4 h			
Time, h	**UV Treatment, s**		
	0	**6**	**18**
0 vs 1	ns	ns	ns
0 vs 2	ns	ns	ns
0 vs 3	ns	ns	ns
0 vs 4	ns	ns	ns

7.3.5. Effect of MAPK inhibition and UV light on PBMC death

7.3.5.1 Effect of JNK inhibition and UV light on PBMC death

The results presented in Section 7.3.4 suggested JNK is not activated in PBMCs following UV light treatment. To provide further evidence to establish the involvement of JNK activation in the response of PBMCs to UV light treatment, PBMCs were treated with SP600125 prior to UV light treatment. There were no differences in PBMC viabilities between PBMCs treated with and without SP600125 for all durations of UV light (Figure 7.7A). Whilst the percentages of apoptotic PBMCs were significantly ($P<0.05$) lower for cells pre-treated with SP600125 compared to PBMCs not treated with SP600125 (Figure 7.7B), the percentages of late apoptotic/secondary necrotic were significantly ($P<0.05$) higher for PBMCs pre-treated with SP600125 (Figure 7.7C). The results suggest SP600125 has minimal effects on UV-induced changes in PBMC viability, providing further support that JNK signalling is not involved in the response of PBMCs to UV light treatment.

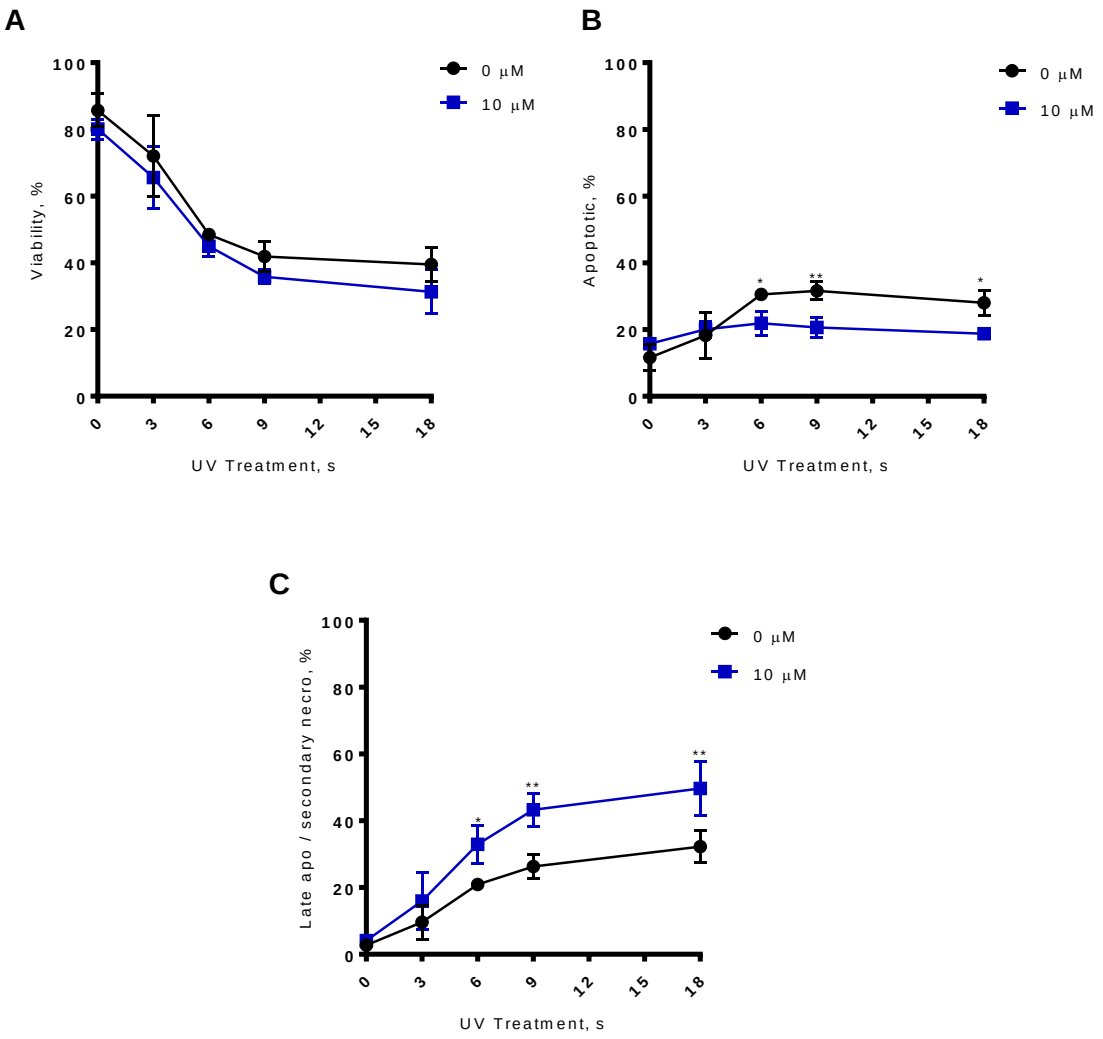

Figure 7.7: Effect of SP600125 and UV on PBMC viability
PBMCs were pre-treated with 0 or 10 µM SP600125 at 37°C for 1 h to inhibit JNK signalling and then treated with various durations of UV light. After 24 h, (A) viability, (B) apoptosis and (C) late apoptosis/secondary necrosis were measured using BD Pharmingen™ FITC Annexin V Apoptosis Detection Kit I. Data presented as mean ± SD, n=3 participants. Experimental measurements were replicated 3 times for each of the participants and the data was combined. Statistical differences shown between SP600125 and no inhibitor at UV time points, calculated using two-way ANOVA with Sidak's *post hoc* test: * (P<0.05), ** (P<0.01).

7.3.4.1 Effect of p38 inhibition and UV light on PBMC death

To determine if p38 signalling is involved in UV-induced cell death in PBMCs, PBMCs were pre-treated with SB202190 prior to UV light treatment. There were no significant differences in PBMC viabilities between PBMCs treated with and without SB202190 for all durations of UV light tested (Figure 7.8A). For PBMCs treated with 3 to 18 s UV, the percentages of apoptotic PBMCs were lower for PBMCs pre-treated with SB202190 compared to PBMCs not treated with SB202190 (Figure 7.8B) whereas the percentages of late apoptotic/ secondary necrotic PBMCs were higher for PBMCs treated with SB202190 (Figure 7.8C). SB202190 therefore causes minimal changes in the different stages of apoptosis but has little effect on the overall viability of the UV-treated PBMCs.

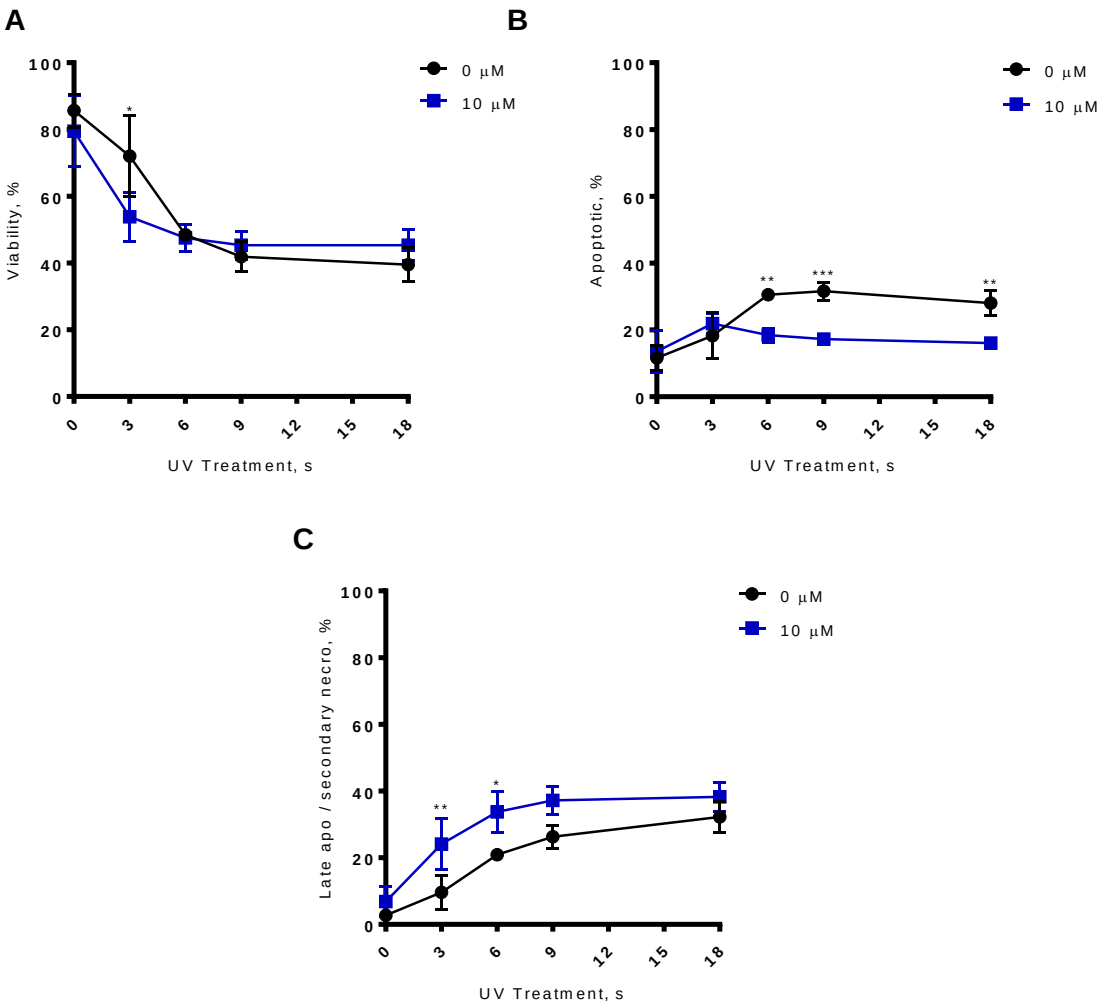

Figure 7.8: Effect of SB202190 and UV on PBMC viability
PBMCS were pre-treated with 0 or 10 µM SB202190 at 37°C for 1 h to inhibit p38 signalling and then treated with various durations of UV light. After 24 h, (A) viability, (B) apoptosis and (C) late apoptosis/secondary necrosis were measured using BD Pharmingen™ FITC Annexin V Apoptosis Detection Kit I. Data presented as mean ± SD, n=3 participants. Experimental measurements were replicated 3 times for each of the participants and the data was combined. Statistical differences shown between SB202190 and no inhibitor at UV time points, calculated using two-way ANOVA with Sidak's *post hoc* test: * (P<0.05), ** (P<0.01), *** (P<0.001).

7.3.6 Effect of vincristine on PBMC death

To determine the effect of vincristine on PBMC death, PBMCs were treated with various concentrations of vincristine and the effect on PBMC viability was assessed using flow cytometry to measure annexin V binding and PI staining. Vincristine treatment had minimal effects on PBMC viability (Figure 7.9A). Although 10 to 40 nM vincristine caused a significant ($P<0.01$) decrease in PBMC viability, viability was only reduced by 5%. The differences observed were due to changes in the percentage of apoptotic cells (Figure 7.9B); the percentages of necrotic and late apoptotic/secondary necrotic cells were unaffected by vincristine treatment (data not shown).

The effects of vincristine on PBMC death was further investigated using higher concentrations of vincristine, 0 to 100 µM. Treatment with 0 to 12.5 µM vincristine had no significant effect on PBMC viability compared to untreated PBMCs (Figure 7.9C). Treatment with 100 µM vincristine caused a significant ($P<0.01$) decrease in PBMC viability compared to untreated PBMCs but only by around 10%. Vincristine treatment had no effect on the percentage of necrotic or late apoptotic/secondary necrotic PBMCs (data not shown), the small but significant difference in PBMC viability following treatment with 100 µM vincristine was due to a significant ($P<0.01$) increase in the percentage of apoptotic PBMCs (Figure 7.9D). Collectively, these results show vincristine does not induce significant levels of cell death in PBMCs, even at high concentrations of vincristine.

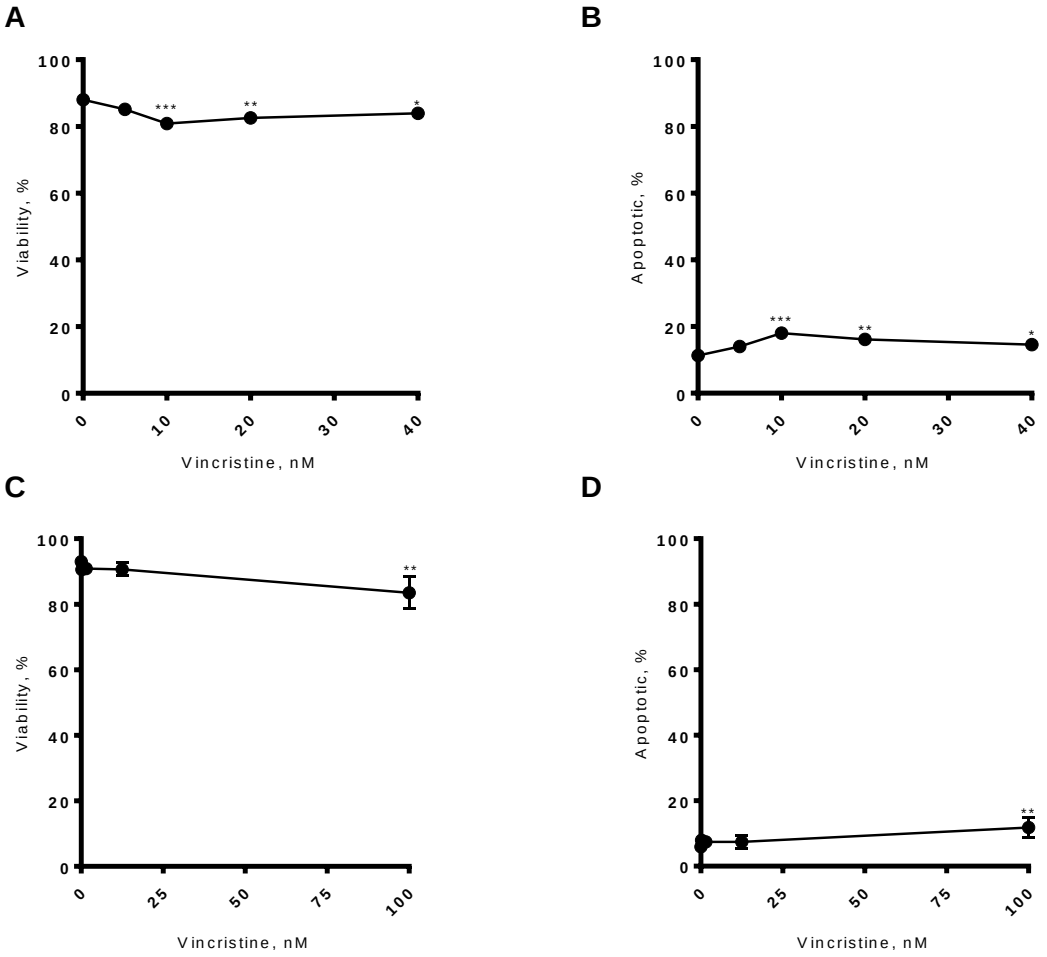

Figure 7.9: Effect of vincristine on PBMC viability
PBMCs were treated (A,B) 0 to 40 nM or (C-D) 0 to 100 nM vincristine and then incubated at 37°C for 24 h. (A,C) viability or (B,D) apoptosis were measured using BD Pharmingen™ FITC Annexin V Apoptosis Detection Kit I. Data presented as mean ± SD, n=3 experimental measurements for a single participant. Statistical differences shown to untreated PBMCs, calculated using one-way ANOVA with Dunnett's *post hoc* test: * (P<0.05), ** (P<0.01), *** (P<0.001).

7.3.6 Effect of MAPK inhibition and vincristine on PBMC death

7.3.6.1 Effect of JNK inhibition and vincristine on PBMC death

The effect of vincristine treatment on the activation of MAPKs in PBMCs was not investigated. However due to the limited ability of vincristine to induce cell death in PBMCs observed in Section 7.3.5, PBMCs were treated with SP600125 prior to vincristine treatment to assess if JNK inhibition altered the susceptibility of PBMCs to vincristine-induced cell death. Whilst significant ($P<0.0001$) differences in PBMC viabilities were observed between PBMCs treated with and without SP600125 and equivalent concentrations of vincristine, the differences were minimal (Figure 7.10A). The differences were due to small alterations in the percentage of apoptotic PBMCs (Figure 7.10B). SP6002125 therefore did not alter the sensitivity of PBMCs to vincristine-induced cell death.

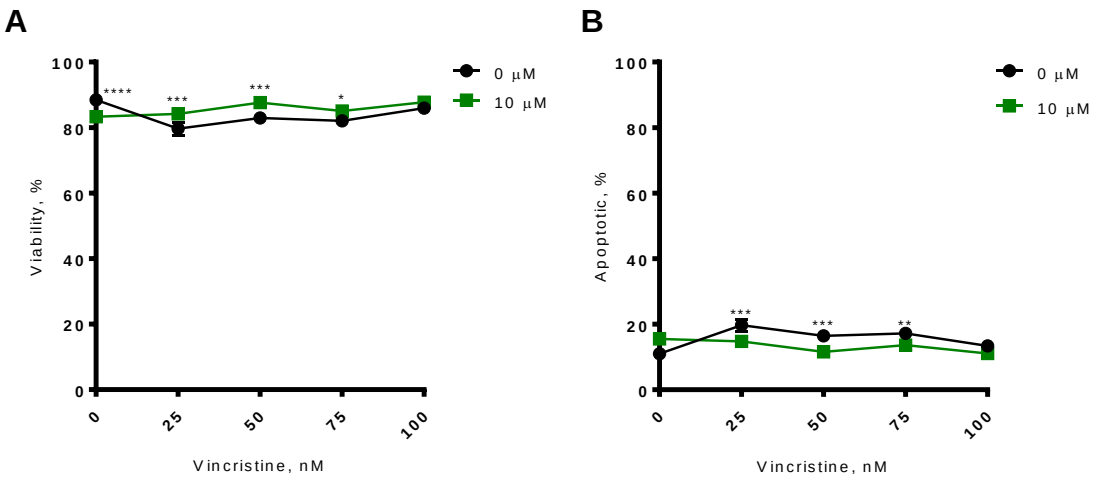

Figure 7.10: Effect of SP600125 and vincristine on PBMC viability
PBMCs were pre-treated with 0 or 10 µM SP600125 at 37°C for 1 h to inhibit JNK signalling and then treated with various concentrations of vincristine. After 24 h, (A) PBMC viability or (B) apoptosis were measured using BD Pharmingen™ FITC Annexin V Apoptosis Detection Kit I. Data presented as mean ± SD, n=3 participants. Experimental measurements were replicated 3 times for each of the participants and the data was combined. Statistical differences shown between SP600125 and no inhibitor at vincristine concentration points, calculated using two-way ANOVA with Sidak's *post hoc* test: * (P<0.05), ** (P<0.01), *** (P<0.001), **** (P<0.0001).

7.3.6.2 Effect of p38 inhibition and vincristine on PBMC death

Further investigations were performed to investigate if inhibition of another MAPK pathway, the p38 pathway, altered the sensitivity of PBMCs to cell death induced by vincristine. The small reductions observed in PBMC viability following vincristine treatment were prevented by inhibition of p38 signalling using SB202190 (Figure 7.11A). The differences were not due to changes in the percentages of necrotic or late apoptotic/secondary necrotic PBMCs (data not shown). The percentages of apoptotic PBMCs were lower for vincristine-treated PBMCs pre-treated with SB202190 compared to those not pre-treated with SB202190 (Figure 7.11B). Although the effects are minimal, it appears that inhibition of p38 signalling, using SB202190 does prevent vincristine-induced changes in PBMC viability.

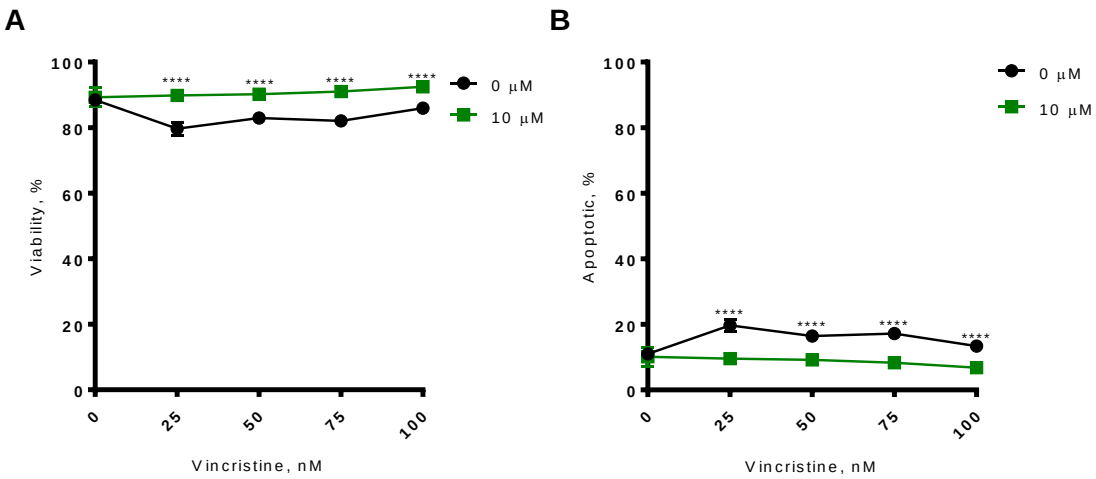

Figure 7.11: Effect of SB202190 and vincristine on PBMC viability
PBMCs were pre-treated with 0 or 10 μM SB202190 at 37°C for 1 h to inhibit p38 signalling and then treated with various concentrations of vincristine. After 24 h, (A) PBMC viability or (B) apoptosis were measured using BD Pharmingen™ FITC Annexin V Apoptosis Detection Kit I. Data presented as mean ± SD, n=3 participants. Experimental measurements were replicated 3 times for each of the participants and the data was combined. Statistical differences shown between SB202190 and no inhibitor at vincristine concentration points, calculated using two-way ANOVA with Sidak's *post hoc* test: **** (P<0.0001).

7.4 Discussion

The overall aim of this chapter was to gain a greater understanding of the role of MAPK signalling in leukemic cell death by comparing the effects observed in previous chapters using the U937 human monocytic cell line, to PBMCs from healthy individuals.

7.4.1 Levels of active MAPKs in PBMCs

The JNK and p38 signalling pathway are typically activated by stress and therefore prior to performing experiments using PBMCs, it was necessary to establish whether or not the process of PBMC isolation had an effect on MAPK activation (Section 7.3.1). PBMC isolation had minimal effects on the activation of JNK, c-Jun and p38 and therefore PBMCs were treated immediately following isolation in the remaining experiments in this chapter.

Low levels of phosphorylated JNK, c-Jun and p38 were measured in untreated PBMCs which is consistent with the observations in U937 cells (Section 3.3.5). Previous studies have demonstrated leukemic cells have high levels of active ERK compared to non-leukemic cells (Milella et al., 2001; Morgan, Dolp, & Reuter, 2001; Ricciardi et al., 2005). The work presented in this thesis is consistent with these previous findings. In chapter 3, it was shown that 95% of untreated U937 cells expressed phosphorylated ERK (Section 3.3.5) whereas only 32% of untreated healthy PBMCs expressed phosphorylated ERK (Section 7.3.1). It is not surprising that a higher percentage of untreated PBMCs express phosphorylated ERK compared to phosphorylated JNK and p38, as ERK signalling is involved in cell survival (Shaul & Seger, 2007; Yoon & Seger, 2006).

7.4.2 Activation of MAPKs in PBMCs

Prior to targeting MAPK signalling in combination with cell stressors in PBMCs, it was necessary to confirm that the concentrations of MAPK inhibitors used in U937 cells were able to achieve MAPK inhibition in PBMCs. This was achieved in Chapter 3 by using the MAPK signalling inhibitors in combination with a signalling agonist, anisomycin (Section 3.3.5). Initially the effects of anisomycin on MAPK activation was tested and results showed treatment with

1 µM anisomycin for 30 min had minimal effects on the activation of JNK, c-Jun, p38 and ERK (Section 7.3.2).

The effect of anisomycin on MAPK activation was investigated further by focusing on the activation of JNK. Initially higher concentrations of anisomycin were investigated but results showed higher concentrations of anisomycin had no effect on the activation of JNK. A 30 min incubation with anisomycin was selected based on the results in U937 cells (Section 3.3.5) but as no effects were observed in PBMCs after this period of incubation, a longer incubation (1 h) with anisomycin was tested but showed no effect on JNK activation. It was believed the lack of JNK activation following anisomycin treatment was unlikely to be due to the methods used to measure phosphorylated MAPKs as phosphorylated ERK was detected in untreated PBMCs (Section 7.3.2). In addition, alternative methods to prepare PBMCs for flow cytometry were tested, such as the as the permeabilisation of PBMCs using BD Phosflow™ Perm Buffer II, but similar results were observed as those presented in this chapter (data not shown).

The results presented in this chapter therefore suggest anisomycin is unable to activate MAPKs, in particular JNK, in PBMCs (Section 7.3.2). This is in contrast to the effects of anisomycin in U937 cells, in which anisomycin treatment readily causes the activation of JNK, c-Jun and p38 (Section 3.3.5). In order to make a direct comparison to U937 cells, the effect of anisomycin specifically on MAPK activation in monocytes needs to be investigated, which could be achieved by enriching the monocytes from the PBMCs.

There appears to be no previous published studies regarding the effects of anisomycin specifically in healthy PBMCs. It was expected anisomycin would be effective in PBMCs as anisomycin is a protein synthesis inhibitor which functions by inhibiting peptidyl transferase during translation in eukaryotic ribosomes (Hori et al., 2008). Cancer cells have a high demand for protein synthesis due to high rates of proliferation and increased rates of survival (Bhat et al., 2015). The difference in the sensitivities of U937 cells and healthy PBMCs to anisomycin could be attributed to different protein synthesis rates between cancer and non-cancer cells. For example, various cancer cell lines were 2-4 times more susceptible to cell death induced by amicoumacin A, an alternative protein synthesis inhibitor, compared to non-cancer cell lines

(Prokhorova et al., 2016). Therefore, alternative compounds to protein synthesis inhibitors, might be more suitable as MAPK signalling agonists in healthy PBMCs. Lipopolysaccharide is a potential compound which has previously been shown to activate MAPKs in PBMCs (van den Blink et al., 2001; Wu, Alexis, Bromberg, Jaspers, & Peden, 2009)

7.4.3 UV light treatment

The effect of UV light on PBMC death was investigated and results showed PBMC viability decreased in a dose-dependent manner (Section 7.3.3) which was similar to that observed in U937 cells (Section 3.3.1). It is well established that UV light is able to activate JNK signalling; UV light was used as an alternative stimulus to induce JNK activation in PBMCs. JNK was activated within 15 min following anisomycin treatment in U937 cells (Section 3.3.5), suggesting JNK is rapidly activated in response to cell stressors. Therefore, the activation of JNK signalling following UV treatment in PBMCs was measured up to 60 min following UV treatment but results showed JNK was not activated during this time (Section 7.3.4). A longer incubation time (up to 4 h) was studied but also showed no activation of JNK. The ability of UV light to induce JNK activation in PBMCs from healthy individuals has however been shown in a previous study; both JNK1 and JNK2 were activated in PBMCs following treatment with 60 J/m^2 UV for 30 and 60 min (Wang et al., 2011). The ability of UV to stimulate JNK activation in PBMCs as observed in the study by Wang et al. (2011) could be due to the longer durations of UV treatment used compared to the short durations used in this thesis. Another possible explanation for the inconsistencies in results could be due to the methods used to measure JNK activation in PBMCs; Wang et al. (20011) used Western blotting whereas flow cytometry was used in this chapter. It was originally believed that the lack of JNK activation was not due to the flow cytometry method used to detect phosphorylated JNK in PBMCs, however additional measurement of JNK phosphorylation by Western blotting in PBMCs following both anisomycin and UV light treatment would have provided clarification of this matter.

As neither anisomycin nor UV light were able to activate MAPK signalling in PBMCs, it was not possible to confirm the effect of MAPK inhibitors on MAPK phosphorylation in PBMC. Therefore, the same concentration of inhibitors, as were used for U937 cells were tested, but without confirmation of signalling inhibition, the conclusions which can be drawn from the following data are limited.

7.4.4 MAPK inhibition and UV light

In Chapter 4, it was shown that inhibition of JNK signalling using SP600125, made U937 cells more susceptible to UV-induced apoptosis (Section 4.3.2.1). In this chapter, PBMCs were treated with SP600125 to determine if JNK inhibition altered the susceptibility of PBMCs to UV-induced cell death (Section 7.3.5.1). The data shown is data combined for 3 individual participants, yet interestingly similar effects were observed in each of the 3 individuals (individual data not shown). SP600125 had little effect on the sensitivity of PBMCs to cell death induced by UV light, suggesting JNK signalling is not involved in the response of PBMCs to UV light. Similar effects on UV light-induced death in PBMCs were observed when p38 signalling was inhibited using SB202190. The effects observed are not as striking as in U937 cells, but without confirmation of the ability of 10 µM SP600125 or 10 µM SB202190 to inhibit JNK and p38 phosphorylation PBMCs respectively, limited valid conclusions can be drawn from the data.

7.4.5 Vincristine treatment

The effect of vincristine on PBMC death was investigated and results showed concentrations of vincristine up to 40 nM, which were effective at inducing cell death in U937 cells (Section 3.3.3.2), had minimal effects on cell death in PBMCs (Section 7.3.6). Higher concentrations of vincristine, up to 100 µM, were tested but these concentrations also had minimal effect on PBMC cell death. These results suggest U937 cells are more susceptible to vincristine-induced cell death than healthy PBMCs. These results are in agreement with a previous study which reported the IC_{50} value for vincristine in U937 cells as 0.09 ng/mL, whereas the IC_{50} value for PBMCs was greater than 100 ng/mL

(Chadarat Ampasavate et al., 2010). As vincristine binds to tubulin and prevents subsequent cell division, the differences in susceptibility of U937 cells and PBMCs to vincristine are likely to be due to differences in proliferation rates; U937 cells have a high proliferation rate whereas *ex vivo* PBMCs in culture have low proliferation rates.

7.4.6 MAPK inhibition and vincristine

Treatment with SP600125 protected U937 cells from vincristine-induced cell death suggesting the importance of JNK activation in the induction of U937 cell death in response vincristine (Section 4.3.4.3). PBMCs were treated with SP600125 in combination with vincristine, to determine if JNK signalling inhibition, altered the susceptibility of PBMCs to cell death (Section 7.3.6.1). Inhibition of JNK signalling, using SP600125 however had minimal effects on vincristine-induced cell death in PBMCs, suggesting JNK signalling is not involved in the protection against PBMC cell death in response to vincristine.

Inhibition of p38 signalling, using SB202190, also protected U937 cells from vincristine-induced cell death (Section 5.3.5). Although the effect of vincristine treatment alone was minimal, inhibition of p38 signalling in PBMCs using SB202190, also protected the PBMCs from vincristine- induced cell death (Section 7.3.6.2). Therefore it appears that the activation of p38 signalling is important for providing protection from vincristine-induced cell death in both U937 cells and PBMCs from healthy individuals. This suggests that potential methods to promote the activation of p38 in leukemic cells in response to vincristine may also increases the levels of p38 and subsequent cell death in non-leukemic cells.

7.5 Summary

In this chapter, the activation of JNK following anisomycin or UV light treatment was not detected. Further work is needed to confirm the reasons for this and to confirm the ability of MAPK inhibitors to inhibit MAPKs in PBMCs. UV light decreased PBMC viability in a similar manner to U937 cells, but the ability of JNK and p38 inhibition to alter the susceptibility of PBMCs to UV light was minimal. PBMCs are less sensitive to vincristine-induced cell death compared

to U937 cells. JNK inhibition had little effect on PBMC death yet p38 inhibition prevented vincristine-induced death.

It is difficult to draw valid conclusions from the work presented in this chapter, as each experiment was conducted on PBMCs from a very limited number of healthy individuals, up to 3, but in some cases, only a single individual. Further work involving a larger number of individuals is required; studying a larger number of individuals will also provide an insight into variation between individuals.

Chapter 8

General Discussion

Chapter 8: General Discussion

The overall aim of this thesis was to investigate the role of MAPK signalling pathways in leukemic cell death. This was achieved by targeting the JNK, p38 and ERK pathways in combination with various cell stressors in the U937 cell line. In addition, preliminary work was performed to investigate the role of MAPK pathways in PBMCs from healthy individuals. The aim of this chapter is to discuss potential models for the involvement of MAPK signalling pathways in leukemic cell death in response to the stimuli tested in this thesis, and to consider the implications of these findings. The approaches taken to target MAPK signalling will be discussed, and areas for potential further work will be identified.

8.1 Role of MAPKs in leukemic cell death

The constitutive activation of ERK1/2 has previously been reported in AML cells (Kim et al., 1999; Milella et al., 2001; Ricciardi et al., 2005), consistent with this, the results presented in this thesis show high levels of phosphorylated ERK1/2 in U937 cells. High levels of active ERK1/2 mediate leukemic cell survival (Kerr et al., 2003; Milella et al., 2001; Morgan et al., 2001), confirmed in this study, where inhibition of ERK1/2 signalling in U937 cells reduced cell survival. As the role of ERK1/2 signalling in leukemogenesis appears to be well-established and less is known about the roles of the JNK and p38 pathways, the JNK and p38 pathways will remain the focus for the following discussion.

JNK signalling has distinct and opposing functions in cancer (Bubici & Papa, 2014; Tournier, 2013) and whilst the role of JNK signalling appears to be well established in solid tumours, the role in leukemic cell death is poorly understood. There is conflicting evidence for the role of JNK signalling in the response of leukemic cells to chemotherapy (Cripe et al., 2002; Lagadinou et al., 2008; Liou et al., 2017; Mc Gee et al., 2002; Nica et al., 2008). p38 signalling has been implicated primarily in lymphocytic leukaemias (Bendall et al., 2005; Gaundar et al., 2009; Ringshausen et al., 2004), whilst the role in myeloid leukaemias, in particular AML, is poorly understood. As for JNK signalling, the role of p38 in the response of leukemic cells to chemotherapy,

is controversial (Dumka et al., 2009; Giafis et al., 2006; Parmar et al., 2004; Pedersen et al., 2002). The role of MAPKs in leukemic cell death therefore appears to be specific to the type of leukaemia, the cell type and the chemotherapy used. This thesis utilised U937 cells, as a model cell line for AML. The role of MAPK pathways in U937 cell death was investigated in this thesis in response to various cell stressors: UV light, heat treatment and the chemotherapeutic agents doxorubicin and vincristine.

One of the most important conclusions from the work presented in thesis is that the role of MAPKs in U937 cell death is stimuli-specific. UV treatment and heat treatment provided a good model for investigating the role of MAPK signalling pathways in leukemic cell death. However the findings that the role of MAPKs in leukemic cell death is stimuli-specific, highlights that future work should focus on investigating the role of MAPKs in response to specific chemotherapeutic agents, as this will have the most important clinical implications. The role of MAPK signalling pathways in the response to UV, heat treatment and doxorubicin in leukemic cells will briefly be discussed here. The most important findings in this thesis are related to vincristine, hence the role of MAPKs in vincristine-induced cell death in leukemic cells will be discussed in more depth.

8.1.1 Role of MAPKs in leukemic cell death induced by UV

Radiation is a type of cancer treatment which induces cell death by causing direct DNA damage and protein damage (Reisz, Bansal, Qian, Zhao, & Furdui, 2014). UV light was used as an apoptotic stimulus in this thesis in order to investigate the role of MAPK signalling pathways in leukemic cell death induced by DNA and protein damage (Figure 8.1). Results of this study showed ERK signalling was not involved in the response of U937 cells to UV-induced cell death, which is in agreement with previous findings in U937 cells (Luchetti et al., 2009). In this thesis, increased UV-induced cell death was observed in U937 cells following inhibition of JNK and p38 signalling. This suggests that the activation of JNK and p38 signalling mediates cell survival in response to DNA and protein damage induced by UV light (Figure 8.1).

Although typically the main function of p38 signalling is to promote cell death, p38 is able to mediate cell survival in certain cellular contexts (Cappellini et al., 2005; Kurosu et al., 2005), including the response to DNA damage. Two cell cycle checkpoints which allow for DNA repair are the G1/S checkpoint and the G2/M checkpoint (Bartek & Lukas, 2007). p38 activation is involved in the activation of both checkpoints, enabling cell survival (Thornton & Rincon, 2009). Activation of p38 is required for the initiation of the G2/M checkpoint following UV treatment (Bulavin et al., 2001). Given the role of p38 activation in the induction of cell cycle checkpoints, it is likely that the p38-mediated survival of U937 cells in response to UV treatment observed in this thesis is due to the role of p38 in facilitating DNA repair mechanisms prior to mitosis. This idea is further supported by the evidence that the activation of MAPKAPK-2, a downstream target of p38, is also required for mediating U937 cell survival in response to UV (Figure 8.1). MAPKAPK-2 has been shown to be critical for the G2/M and G1/S checkpoints following UV-induced DNA damage (Manke et al., 2005). In order to confirm that p38 activation mediates cell survival in U937 cells following UV treatment by activating cell cycle checkpoints, the effect of p38 inhibition and UV light treatment on the activation of checkpoint kinases, such as checkpoint kinase 1 (Chk1) and checkpoint kinase 2 (Chk2) could be measured. The p53 tumour suppressor protein and p21 protein are also likely to be involved; it would therefore be useful to also measure the effects of p38 inhibition and UV light treatment on the activation of p53 and p21.

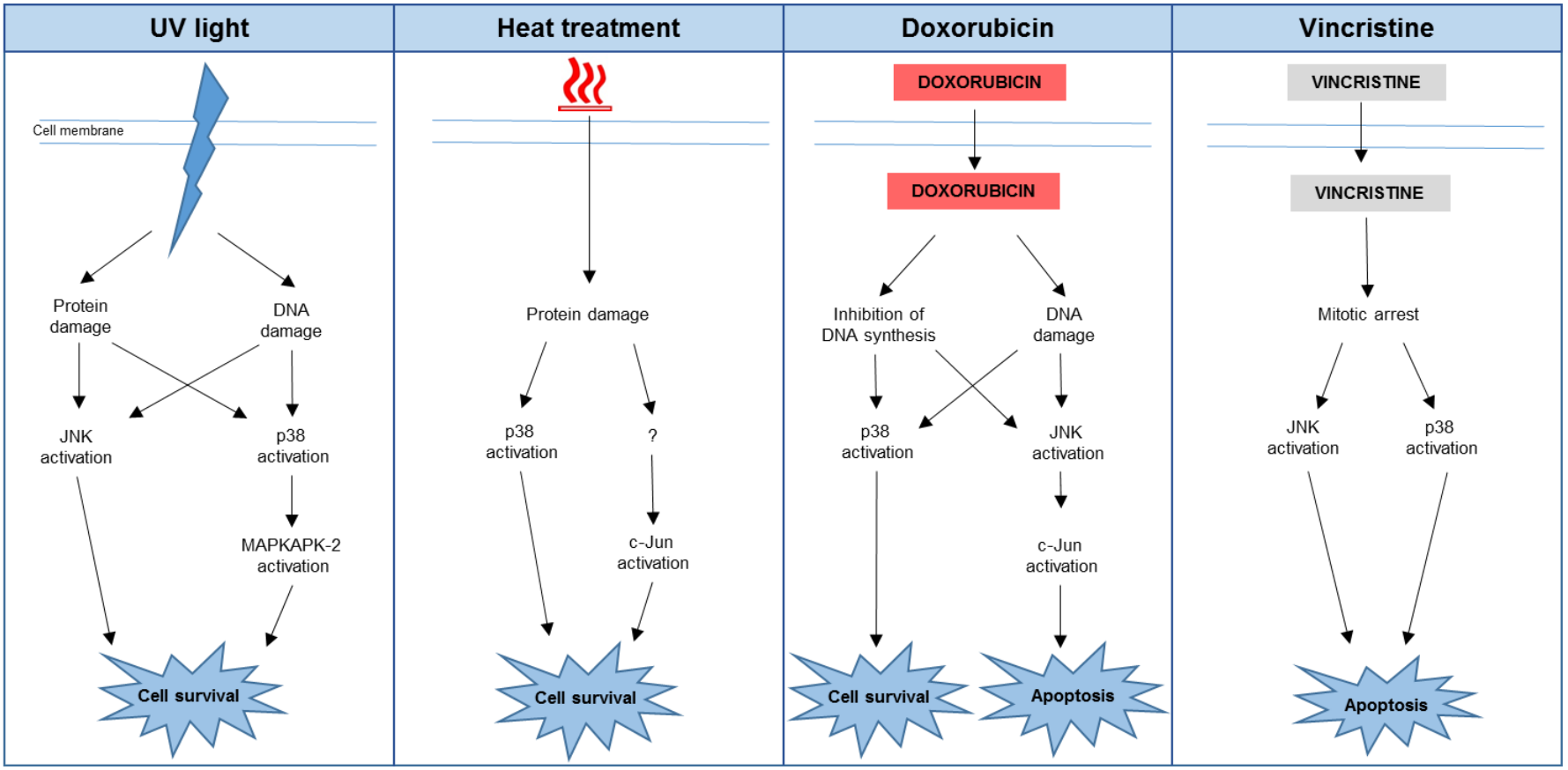

Figure 8.1: Model for the involvement of MAPK signalling pathways in the response of U937 cells to various cell stressors
The JNK and p38 signalling pathways are involved in mediating either cell survival or apoptosis in response to UV light, heat treatment, doxorubicin and vincristine. The activation of JNK and p38 is essential for mediating cell survival in response to UV light. Cell survival in response to heat treatment is mediated by p38 activation and activation of c-Jun by a pathway which does not require JNK activation. p38 activation mediates cell survival in response to doxorubicin treatment, whereas JNK activation is required for induction of apoptosis. The activation of JNK and p38 is required for vincristine-induced apoptosis.

The role of JNK signalling in the response to UV treatment is however somewhat surprising. The work presented in this thesis suggests that the activation of JNK, but not c-Jun is important for mediating U937 cell survival in response to UV treatment. These findings contradict a vast amount of previous research which implicates the apoptotic role of JNK signalling, particularly in the response to UV treatment. There are two possible explanations which could explain these observations. Firstly, the conclusion that the activation of JNK mediates cell survival is based on data using the JNK inhibitor, SP600125. The effects of JNK inhibition and UV light treatment on the activation of JNK and c-Jun were not directly measured. It is possible the results observed cannot be attributed to direct alterations in JNK activation. SP600125 was initially believed to be a highly specific inhibitor of JNK signalling (Bennett et al., 2001), others have argued that although SP600125 shows greater inhibition of JNK phosphorylation compared to other kinases, SP600125 is able to inhibit other kinases such as p38 to an extent (Bain, Plater, et al., 2007; Bain, Mclauchlan, Elliott, & Cohen, 2003). Therefore it is possible that the effects observed are due to the effects of SP600125 on p38 signalling and hence the findings are similar to those previously discussed or an alternative pathway may be affected.

The alternative explanation is that SP600125 does inhibit JNK phosphorylation but the inhibition of the JNK signalling pathway has effects on other signalling pathways. Signalling pathways do not act in isolation, but rather function as part of a complex signalling network with interactions between signalling pathways, termed cross-talk. Cross-talk between signalling pathways ensures that the correct cellular response is triggered in response to a stimulus. One such example is the cross-talk between the p38 and JNK signalling pathways; cross talk between p38 and JNK, mediated by MAPK phosphatase-1 (DUSP1/MKP-1), is important for determining the fate of the cell following exposure to UV light (Staples, Owens, Maier, Cato, & Keyse, 2010). Cross-talk however does not just occur between two pathways, multiple pathways are involved. If JNK is unable to be activated following UV-induced DNA damage, cross-talk may enable alternative pathways to be activated, which induce cell death. Surprisingly, a highly specific inhibitor of c-Jun

phosphorylation was utilised and showed c-Jun has no effect on UV-induced cell death in U937 cells. If JNK signalling is involved in UV-mediated cell survival, it is possible other downstream targets of JNK phosphorylation are activated when c-Jun activation is inhibited; a single kinase is capable of activating multiple downstream targets. To investigate these two theories, the effects of JNK inhibition and UV light treatment on the components of the JNK signalling pathway and alternative pathways need to be measured.

Results also showed that PBMCs from healthy individuals were as susceptible to UV treatment as the U937 cells. This has important clinical implications as it confirms radiation is not a suitable treatment for leukaemia, as both leukemic and non-leukemic cells would be susceptible to the radiation. As leukemic cells do not form a solid tumour, it would be difficult to selectively target the leukemic cells with radiation. This, however, is a general phenomenon for the treatment of cancer with radiation; hence chemotherapies which specifically target cancer cells are a more desirable treatment option. The JNK and p38 signalling pathways provided protection from UV-induced cell death in PBMCs, as observed for U937 cells. This suggests that the involvement of JNK and p38 signalling in providing protection against UV-induced cell death is a general response and is not specific to leukemic cells.

8.1.2 Role of MAPKs in leukemic cell survival in response to heat treatment

Sub-lethal heat treatments, which alone are insufficient to cause cell death, are able to sensitise cancer cells to radiation therapy and chemotherapy (Falk & Issels, 2001; Hildebrandt et al., 2002; Jolly & Morimoto, 2000). Heat treatment causes protein damage (Figure 8.1) as small increases in temperature cause protein unfolding and protein aggregation. Protein damage as a result of heat treatment initiates a cellular response, termed the heat shock response. The heat shock response involves the upregulation of a family of proteins, the heat shock proteins (HSPs) which function as molecular chaperones and facilitate the refolding of damaged proteins. The HSPB1 (HSP27), HSPA (HSP70) and HSPC (HSP90) subfamilies have a protective

role, preventing apoptosis induced by various stimuli, including heat treatment (Beere, 2005).

Although ERK signalling plays an important role in cell survival, results showed that ERK activation was not involved in mediating cell survival in U937 cells in response to heat treatment. It was however shown that the activation of p38 is required for the survival of U937 cells following heat treatment (Figure 8.1). This is not surprising as HSPB1 is phosphorylated by MAPKAPK-2, a downstream target of p38 (Cuenda et al., 1995). Although the results of this study do not suggest an involvement of MAPKAPK-2 in the response of U937 cells to heat treatment, this is more likely to be due to lack of inhibition of MAPKAPK-2 by SB203580 as previously discussed. It is likely that protein damage, as a result of heat treatment causes the activation of p38, the subsequent activation of MAPKAPK-2 and the phosphorylation of HSPB1. HSPB1 is a signalling molecule which has multiple downstream targets. One mechanism by which HSPB1 could promote cell survival is by interacting with protein kinase B (Akt). The interaction between Akt and HSPB1 stabilises Akt which is a kinase that promotes cells survival (Rane et al., 2003). This interaction may be mediated by HSPC proteins (Sato, Fujita, & Tsuruo, 2000). HSPB1 could also prevent apoptosis by preventing the release of cytochrome c (Paul et al., 2002). These are just two possible mechanisms by which p38-mediated activity of HSPB1 could promote cell survival in U937 cells following heat treatment. The anti-apoptotic role of HSPs is too extensive to discuss further here. It is however evident that the interactions between HSPs and MAPK signalling pathways in leukemic cells needs confirming.

The work presented in this thesis suggests the activation of JNK is not involved in the response of U937 cells to heat treatment. HSPA proteins are anti-apoptotic proteins which are able to inhibit the phosphorylation and activation of JNK (Li et al., 2010; Mosser et al., 2000; Park, Lee, Huh, Seo, & Choi, 2001). It is possible that in U937 cells, HSPA proteins are induced following heat treatment, which inhibit the activation of JNK, and hence no further effects are observed when the cells are treated with SP600125. The activation of c-Jun is however important for U937 cell survival following heat treatment (Figure 8.1). c-Jun is typically activated by JNK signalling, but as results suggest JNK is not activated following heat treatment, it is possible c-

Jun is activated by an alternative pathway. The mechanism by which c-Jun is activated following heat treatment requires further investigation.

8.1.3 Role of MAPKs in leukemic cell death induced by doxorubicin

Doxorubicin, an anthracycline antibiotic, is a chemotherapy used in the treatment of leukaemia. Doxorubicin exerts its intracellular effects on cell death through two mechanisms: inhibition of DNA synthesis and DNA damage (Figure 8.1). Doxorubicin directly intercalates with DNA and it is the interaction between doxorubicin and DNA which inhibits DNA topoisomerase II and prevents the synthesis of DNA (Yang et al., 2014). Alternatively, upon entering a cell doxorubicin can become oxidised by proteins in the cytoplasm to form a doxorubicin semiquinone. The doxorubicin semiquinone is an unstable metabolite which is readily reduced back to doxorubicin by a process which generates reactive oxygen species (ROS), such as free radicals. Generation of ROS results primarily in DNA damage, but also protein damage and lipid peroxidation, all of which trigger apoptosis.

The activation of stress-activated protein kinases following doxorubicin treatment is well established in cancer cells (Kim & Freeman, 2003; Krilleke et al., 2003; Lagadinou et al., 2008; Panaretakis et al., 2005). The results presented in this thesis show that the activation of JNK is required for doxorubicin-induced apoptosis in U937 cells (Figure 8.1), which is consistent with previous findings (Lagadinou et al., 2008). The pro-apoptotic role of c-Jun, a downstream target of JNK has previously been implicated in the response to doxorubicin; Lagadinou et al. (2008) however did not investigate the involvement of c-Jun in U937 cells. In this thesis it was shown that the activation of c-Jun is also important for the apoptotic effects of doxorubicin in U937 cells (Figure 8.1). It is widely believed that JNK signalling is involved in the induction of the intrinsic (mitochondrial) apoptotic pathway. In U937 cells, the activation of JNK and c-Jun following DNA damage and inhibition of DNA synthesis is likely to activate pro-apoptotic proteins (Bak, Bax and BH3-only proteins). This results in the release of cytochrome c from the mitochondria, activation of effector caspases and subsequent activation of apoptosis. Although the downstream effects of JNK signalling activation on apoptosis

appear to be well-characterised, the mechanisms by which doxorubicin leads to JNK activation requires further investigations.

Both doxorubicin and UV light cause DNA damage yet the involvement of JNK signalling in response to the two stressors is different and opposing. This could be related to the mechanisms by which DNA damage occurs. UV light has a direct effect on DNA, whereas DNA damage induced by doxorubicin is an indirect effect as a result of the generation of ROS. Alternatively, doxorubicin exerts its apoptotic effects by two mechanisms, inhibition of DNA synthesis and causing DNA damage. It is possible that JNK activation plays a more prominent role in the induction of apoptosis following inhibition of DNA synthesis, whereas p38 is involved in apoptosis induced by DNA damage, or vice versa.

In contrast to JNK signalling, p38 is activated in U937 cells following doxorubicin treatment and the activation of p38 is important for promoting cell survival in response to doxorubicin (Figure 8.1). This observation could be related to the involvement of p38 in cell cycle checkpoints, as discussed for UV light (Section 8.1.1).

8.1.4 Role of MAPKs in vincristine-induced cell death

Microtubule-targeting agents, such as the vinca alkaloids (vincristine, vinblastine) are commonly used in the treatment of various types of leukaemia. Microtubules are intracellular filaments which have an important function in chromosomal segregation during mitosis (cell division), which makes them an ideal target for chemotherapy (Dumontet & Jordan, 2010; Jordan & Wilson, 2004). Vincristine inhibits microtubule formation by binding to the β-tubulin subunit of the α-β heterodimer and preventing the addition of tubulin dimers to the assembly end (+ end) of the microtubule (Figure 8.2). Inhibition of microtubule assembly prevents mitotic spindle formation, disables the movement and alignment of chromosomes and hence blocks progression from G2 to M phase of the cell cycle. A vast amount of previous research has implicated MAPK signalling pathways in the response of cancer cells to microtubule inhibitors (Fan & Chambers, 2001), and in particular in the response of leukemic cells to vincristine (Alsadeq et al., 2015; Barančík et al., 2001; Bates et al., 2015a; Shiah et al., 2001; Yoshida et al., 2011). Studies

regarding the role of MAPK signalling pathways in response to microtubule inhibitors are however limited in AML cells. Vinblastine, an alternative microtubule inhibitor to that used in this thesis, induces apoptosis in ML-1 cells (AML cell line) and causes the activation of JNK and p38 in ML-1 cells (Stadheim, Xiao, & Eastman, 2001). However it was not shown if the activation of JNK and p38 was essential for cell death. The results presented in this thesis suggest that the activation of the JNK and p38 signalling pathways are required for vincristine-induced cell death in U937 cells (Figure 8.2). This is likely to occur via a mechanism which does not require the activation of c-Jun and MAPKAPK-2, which are downstream targets of JNK and p38 phosphorylation, respectively. This appears to be the first evidence to link the JNK and p38 signalling pathways to vincristine-induced cell death specifically in AML cells. Further work is however required to confirm that the effects observed are due to changes in the levels of active JNK and p38; signalling inhibitors were used to target the JNK and p38 signalling pathways but the effect of JNK and p38 inhibition and vincristine treatment on the activation of JNK and p38, respectively was not directly measured.

Activation of JNK following vincristine treatment could be due to the accumulation of cells in the G2/M phase of the cell cycle. In normal cells, JNK is temporarily activated during the late G2/M phase of the cell cycle (Fan & Chambers, 2001). Microtubule inhibitors cause accumulation of cells in the G2/M phase, and hence an accumulation of cells expressing JNK; the transient expression of active JNK therefore becomes sustained. The duration of JNK activation depends on the pro- or anti-apoptotic function of JNK (Liu & Lin, 2005) and therefore the sustained activation of JNK as a result of mitotic arrest could result in apoptosis (Chen, Wang, Templeton, Davis, & Tan, 1996). This provides one possible explanation for the involvement of JNK in the response of U937 cells to vincristine. However as p38 signalling was also found to be activated following vincristine treatment, it is more likely that JNK and p38 are activated as part of a stress response induced by the disruption of microtubule formation. JNK and p38 signalling have previously been implicated in the stress response to microtubule disarray (Wang et al., 1998).

In order to fully understand the importance of JNK and p38 activation in vincristine-induced cell death in leukemic cell death, it is necessary to

understand how the pathways are affected in non-leukemic cells. This was investigated in this study using PBMCs from healthy individuals. U937 cells were shown to be more sensitive to vincristine-induced cell death than healthy PBMCs. This is consistent with previous findings in a study also utilising U937 cells and PBMCs from healthy individuals (Ampasavate et al., 2010). This is a very important finding as it indicates vincristine is an ideal therapy as it affects leukemic cell death, whilst having minimal effects on non-leukemic cells. However, in order to draw such conclusions, further work is required to gain a more thorough understanding of the mechanisms which account for the resistance of healthy PBMCs to vincristine.

A possible explanation for the lack of vincristine-induced cell death in healthy PBMCs could be attributed to the differences between transformed and primary cells. Transformed cells are able to rapidly proliferate *in vitro*; as vincristine targets microtubule formation and prevents subsequent cell division, transformed cells such as U937 cells are highly sensitive to vincristine. Healthy PBMCs however are likely to have a much slower rate of proliferation *in vitro;* PBMCs are terminally differentiated cells which have very limited division potential and mitogenic stimuli required for proliferation are likely to be lacking. If the PBMCs are not proliferating there will be a limited requirement for the production of new microtubules and hence there is a lack of target for vincristine (Figure 8.2). In order to establish if the differences observed are due to differences between transformed and primary cells, PBMCs from AML patients need to be compared to PBMCs from healthy individuals. A previous *in vitro* study compared primary leukemic and non-leukemic cells; lymphocytes from healthy individuals were more resistant to vincristine than lymphocytes from children with ALL (Kaspers et al., 1991). However it cannot be assumed that AML cells would behave in a similar manner to ALL cells.

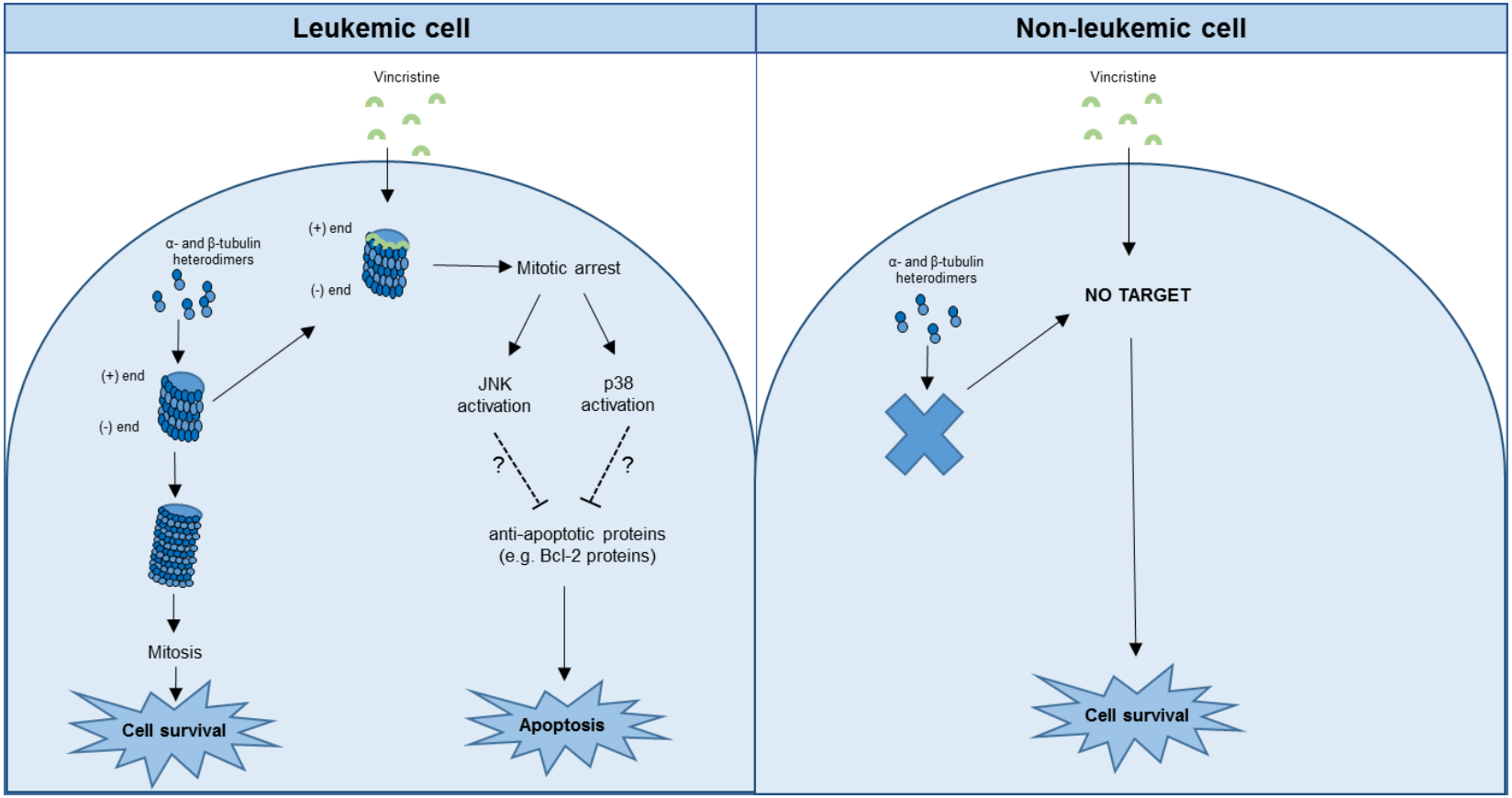

Figure 8.2: Model for the role of JNK and p38 signalling in vincristine-induced apoptosis in leukemic and non-leukemic cells
Microtubules have an important function in chromosomal segregation during mitosis which is critical for the survival of leukemic cells. Microtubules are formed by the addition of tubulin heterodimers to the + end of microtubules. Vincristine inhibits microtubule formation by binding the β-tubulin subunit of the heterodimer, preventing the formation of microtubules. Leukemic cells such as U937 cells have high proliferation rates and inhibition of microtubule formation by vincristine causes mitotic arrest. This causes the activation of JNK and p38, which ultimately leads to apoptosis, through a mechanism which could involve the inhibition of anti-apoptotic proteins. Non-leukemic cells, such as PBMCs do not rapidly proliferate *in vitro* so there is no requirement for new microtubule formation and hence there is no target for vincristine allowing the cells to survive.

In this study, U937 cells, a monocytic cell line was compared to PBMCs, which is a population of cells consisting mainly of monocytes (10-30%) and lymphocytes (70-90%). In addition to different cell types, different populations of cells are likely to present in terms of other factors such as age of the cell. Different populations of cells might have different proliferation rates and be differentially affected by vincristine. In order to allow for a more direct comparison between U937 cells and non-leukemic cells, further investigation should be performed on monocytes isolated from PBMCs.

Although cell lines provide a good model, they do not allow for consideration to variation between individuals. Previous work in a University of Chester laboratory has highlighted the variation in the response of CLL patients to various chemotherapeutics, which has been associated with altered activation of signalling pathways (Dempsey, unpublished data). An alternative study has highlighted variation in the levels of JNK activation following vincristine treatment between patients with CLL (Bates, Lewis, Eastman, & Danilov, 2015). Therefore primary leukemic cells ultimately need to be compared to non-leukemic cells from healthy individuals. Bates et al. (2015) also showed that the activation of JNK in CLL lymphocytes following vincristine treatment was higher for lymphocytes treated *ex vivo* compared to JNK activation in lymphocytes treated *in vivo.* This is important to take into account when considering the clinical implications of this research.

8.2 Targeting MAPK signalling in leukemic cells

One of the main limitations of this study is that the JNK, p38 and ERK pathways were targeted individually and were mainly considered as separate, unrelated pathways. Signalling pathways form complex signalling networks with cross-talk between pathways; future research in this area should take a more integrated approach to targeting the MAPK pathways. Due to the complexity of cell signalling, this would be challenging but there are two possible approaches which could be taken to address signalling cross-talk. As previously mentioned, the effects of MAPK inhibition and cell stressors on MAPK activation need to be assessed in future work. To account for cross-talk between pathways, it is important to not only measure the effects on the

signalling molecule being targeted, but also to simultaneously assess the effects on other signalling molecules following treatment. There are a number of commercial signalling arrays available which can achieve this; these arrays provide a better overview of a signalling response following treatment by showing phosphorylation changes in multiple interrelated pathways. Another approach which could be taken is to use multiple signalling inhibitors simultaneously to target a small number of pathways. The results from a signalling array would direct which pathways would be most appropriate to target. It might be necessary to use multiple inhibitors at different times. It is possible that the inhibition of one pathway may lead to the activation of another, which could then be targeted with an inhibitor following the initial inhibitor. Leukaemia therapy which targets multiple signalling pathways is more likely to be effective than therapy targeting a single pathway.

In this thesis, the JNK, p38 and ERK signalling pathways were targeted using small molecule inhibitors. One of the main strengths of the work presented is that each MAPK signalling pathway was investigated using two alternative inhibitors, which provides a more thorough understanding of the role of each pathway, and in particular the involvement of particular components of the pathway. For example, JNK signalling was targeted using both SP600125 which prevents the phosphorylation of JNK and JNK-IN-8 which prevents the phosphorylation of the downstream target c-Jun.

Targeting MAPK signalling pathways using small molecule inhibitors however presents many problems including poor efficacy and specificity, reversibility and poor selectivity for different MAPK isoforms. With the exception of JNK-IN-8, the MAPK inhibitors used in this thesis were ATP-competitive inhibitors. The efficacy of ATP-competitive inhibitors is reduced by high levels of intracellular ATP (Bogoyevitch, 2005; Bubici & Papa, 2014). In addition, ATP-competitive inhibitors display poor specificity as they target the ATP-binding site which is highly conserved in protein kinases. Although ATP-competitive MAPK inhibitors have been used extensively in research to target MAPK signalling pathways, novel MAPK inhibitors which show greater specificity have been developed and should be utilised in future research. Further research should also focus on the development of MAPK inhibitors

which can be used clinically, and also on methods to deliver these inhibitors specifically to cancer cells.

Not only is specificity a problem with MAPK inhibitors, reversibility of the MAPK inhibitors also limits their use. SP00125 for example is reversible however JNK-IN-8 which is a novel irreversible inhibitor was also tested in this thesis. Where possible, irreversible inhibitors should be used in the future to provide more effective signalling inhibition. Consideration must also be paid to the order in which cells are treated with MAPK inhibitors and cell stressors. Cells were pre-treated with a MAPK inhibitor prior to treatment with a cell stressor. This approach was selected based on previous research and the assumption that addition of the inhibitors prior to treatment would prevent the MAPKs from being activated by the treatments. However in order to gain a more thorough understanding, addition of the inhibitors after treatment should also be tested. This is particularly important for reversible inhibitors, where the treatment with cell stressors has the potential to reverse the effects of the inhibitors.

Given the differential role of MAPK isoforms in cancer, it is important to selectively target individual MAPK isoforms in leukemic cells. It is difficult to achieve this with small molecule inhibitors; the small molecule inhibitors used in this thesis target multiple MAPK isoforms. Isoform-specific targeting of MAPKs can be achieved by knocking down individual MAPK genes using methods to silence genes such as small interfering RNAs (siRNAs). Knockdown of individual MAPK isoforms is an important research tool to identify the importance of a particular isoform. However this approach cannot be taken to target MAPK signalling pathways therapeutically due to the long term and unwanted side effects of the complete loss of a particular MAPK isoform. Not only should individual MAPK isoforms be targeted, but the effects of treatment on individual MAPK isoforms should be measured using isoform-specific antibodies.

8.3 Implications of the research

Collectively, the evidence presented in this thesis suggests that targeting MAPK signalling pathways in combination with chemotherapy has the potential to be an effective therapeutic approach for leukaemia. Some

chemotherapeutic agents require the activation of MAPKs for the induction of cell death, and if a cancer cell has mutations that impair MAPK activation, it will be chemoresistant. Understanding the mechanisms leading to the failure of MAPK activation will be of great therapeutic value, and approaches to enhance the activation of the MAPK required, might prove to be effective. In contrast, where MAPK activation mediates cell survival in response to a chemotherapeutic agent, inhibition of the pathway in combination with standard chemotherapy might be beneficial.

The importance of MAPKs in the development of leukaemia suggests that they might be a useful prognostic marker. High levels of phosphorylated ERK2 has been associated with a poor prognosis in AML patients (Kornblau et al., 2006). Measurement of active MAPK levels in a leukaemia patient prior to commencing treatment might also be beneficial in predicting if a patient will respond to a particular treatment. For example, vincristine-induced cell death requires the activation of JNK. If an individual already has high levels of active JNK prior to starting vincristine treatment, then it is likely they will not respond well to the treatment. Measurement of MAPK levels could therefore be useful in determining which treatment is best to use for a patient. Measurement of MAPK levels could also be useful for monitoring the response of a patient to a treatment. For example, if neither JNK nor p38 are activated following vincristine treatment, this might suggest the patient will not respond to this treatment and an alternative therapy should be started.

The development of the hallmarks of cancer concept has been successful in leading to many generalised therapeutic approaches. However, more recent research, including that previously discussed, suggest inclusion of a personalised elements into the ‘hallmarks’ would be beneficial. In oncology, personalised medicine is the tailoring of medical treatment to the individual characteristics of each patient. This approach relies on the understanding of an individual’s molecular and genetic profile. Here, it has been suggested how an understanding of the MAPK signalling pathways in leukemic cell death could be used to deliver personalised medicine in leukaemia patients. The MAPK signalling pathways however represent a small number of pathways which function as part of a complex signalling network. Gaining a greater understanding of which pathways are deregulated in relation to each of the

hallmarks of cancer will enable a personalised approach to treatment. If a patient presents with certain characteristic, then treatments which target specific signalling pathways related to these characteristics, can be utilised.

Chapter 9

References

Chapter 9: References

Abe, H., Kikuchi, S., Hayakawa, K., Iida, T., Nagahashi, N., Maeda, K., … Laquerre, S. G. (2011). Discovery of a Highly Potent and Selective MEK Inhibitor: GSK1120212 (JTP-74057 DMSO Solvate). *ACS Medicinal Chemistry Letters*, *2*, 320–324.

Abe, M. K., Kahle, K. T., Saelzler, M. P., Orth, K., Dixon, J. E., & Rosner, M. R. (2001). ERK7 Is an Autoactivated Member of the MAPK Family. *Journal of Biological Chemistry*, *276*, 21272-21279.

Admoni-Elisha, L., Nakdimon, I., Shteinfer, A., Prezma, T., Arif, T., Arbel, N., … Shoshan-Barmatz, V. (2016). Novel biomarker proteins in chronic lymphocytic leukemia: Impact on diagnosis, prognosis and treatment. *PLoS ONE*, *11*, 1–28.

Ahmadzadeh, A., Shahrabi, S., Jaseb, K., Norozi, F., Shahjahani, M., Vosoughi, T., … Saki, N. (2014). BRAF mutation in hairy cell leukemia. *Oncology Reviews*, *8*, 50-53.

Ajenjo, N., Aaronson, D. S., Ceballos, E., Richard, C., Leó, J., & Crespo, P. (2000). Myeloid Leukemia Cell Growth and Differentiation Are Independent of Mitogen-activated Protein Kinase ERK1/2 Activation. *The Journal of Biological Chemistry*, *275*, 7189–7197.

Akella, R., Moon, T. M., & Goldsmith, E. J. (2008). Unique MAP Kinase binding sites. *Biochimica et Biophysica Acta* , *1784*, 48–55.

Akincilar, S. C., Unal, B., & Tergaonkar, V. (2016). Reactivation of telomerase in cancer. *Cellular and Molecular Life Sciences*, *73*, 1659–1670.

Alarcon-Vargas, D., & Ronai, Z. (2004). c-Jun-NH_2 Kinase (JNK) Contributes to the Regulation of c-Myc Protein Stability. *Journal of Biological Chemistry, 279,* 5008-5016.

Alessi, D. R., Cuenda, A., Cohen, P., Dudleyţ, D. T., & Saltielţ, A. R. (1995). PD 098059 Is a Specific Inhibitor of the Activation of Mitogen-activated Protein Kinase Kinase in Vitro and in Vivo. *The Journal of Biological Chemistry*, *270*, 27489–27494.

Allen, L. F., Sebolt-Leopold, J., & Mever, M. B. (2003). CI-1040 (PD184352), a targeted signal transduction inhibitor of MEK (MAPKK). *Seminars in Oncology*, *30*, 105–106.

Alsadeq, A., Strube, S., Krause, S., Carlet, M., Jeremias, I., Vokuhl, C., … Schewe, D. M. (2015). Effects of p38α/β inhibition on acute lymphoblastic leukemia (ALL) proliferation and survival *in vivo*. *Leukemia*, *29*, 2307–2316.

Alsayed, Y., Uddin, S., Mahmud, N., Lekmine, F., Kalvakolanu, D. V., Minucci, S., … Platanias, L. C. (2001). Activation of Rac1 and the p38 Mitogen-activated Protein Kinase Pathway in Response to All-trans-retinoic Acid. *Journal of Biological Chemistry, 276,* 4012-4019.

Ampasavate, C., Okonogi, S., & Anuchapreeda, S. (2010). Cytotoxicity of extracts from fruit plants against leukemic cell lines. *African Journal of Pharmacy and Pharmacology*, *4*, 13–21.

Ampasavate, C., Okonogi, S., & Anuchapreeda, S. (2010). Cytotoxicity of extracts from fruit plants against leukemic cell lines. *African Journal of Pharmacy and Pharmacology*, *4*, 13–21.

Arnulf, B., Villemain, A., Nicot, C., Mordelet, E., Charneau, P., Kersual, J., … Hermine, O. (2002). Human T-cell lymphotropic virus oncoprotein Tax represses TGF-□1 signaling in human T cells via c-Jun activation: a potential mechanism of HTLV-I leukemogenesis. *Blood*, *100*, 4129–4138.

Aronov, A. M., Tang, Q., Martinez-Botella, G., Bemis, G. W., Cao, J., Chen, G., … Zie, X. (2009). Structure-Guided Design of Potent and Selective Pyrimidylpyrrole Inhibitors of Extracellular Signal-Regulated Kinase (ERK) Using Conformational Control. *Journal of Medicinal Chemistry*, *52*, 6362–6368.

Arrizau, E., Pippa, R. ., & Oder, M. D. (2016). Protein Phosphatase 2A as a Therapeutic Target in Acute Myeloid Leukemia. *Frontiers in Oncology*, *6*, 1–11.

Ascierto, P. A., Schadendorf, D., Berking, C, ., Agarwala, S. S., van Herpen, C. M. L., Queirolo, P., … Dummer, R. (2013). MEK162 for patients with advanced melanoma harbouring NRAS or Val600 BRAF mutations: A non-randomised, open-label phase 2 study. *The Lancet Oncology*, *14*, 249–256.

Avery, S. V. (2011). Molecular targets of oxidative stress. *Biochemical Journal*, *434*, 201–210.

Bagrodia, S., Dé, B., Davis, R. J., & Cerione, R. A. (1995). Cdc42 and PAK-mediated Signaling Leads to Jun Kinase and p38 Mitogen-activated Protein Kinase Activation. *Journal of Biological Chemistry*, *270*, 27995–27998.

Bain, J., Mclauchlan, H., Elliott, M., & Cohen, P. (2003). The specificities of protein kinase inhibitors : an update. *Biochemical Journal*, *371*, 199–204.

Bain, J., Plate, L., Elliott, M., Shpiro, N., Hastie, J., Mclauchlan, H., … Cohen, P. (2007). The selectivity of protein kinase inhibitors: a further update. *Biochemical Journal* , *15*, 297–315.

Barančík, M., Boháčová, V., Kvačkajová, J., Hudecová, S., Križanová, O., & Breier, A. (2001). SB203580, a specific inhibitor of p38-MAPK pathway, is a new reversal agent of P-glycoprotein-mediated multidrug resistance. *European Journal of Pharmaceutical Sciences*, *14*, 29–36.

Barone, F. C., Irving, E. A., Ray, A. M., Lee, J. C., Kassis, S., Kumar, S., … Parsons, A. A. (2001). SB 239063, a Second-Generation p38 Mitogen-Activated Protein Kinase Inhibitor, Reduces Brain Injury and Neurological Deficits in Cerebral Focal Ischemia. *The Journal of Pharmacology and*

Experimental Therapeutics , *296*, 312–321.

Barr, R. K., Kendrick, T. S., & Bogoyevitch, M. A. (2002). Identification of the critical features of a small peptide inhibitor of JNK activity. *The Journal of Biological Chemistry*, *277*, 10987–10997.

Bartek, J., & Lukas, J. (2007). DNA damage checkpoints: from initiation to recovery or adaption. *Current Opinion in Cell Biology*, *19*, 238–245.

Bates, D. J. P., Lewis, L. D., Eastman, A., & Danilov, A. V. (2015). Vincristine activates c-Jun N-terminal kinase in chronic lymphocytic leukaemia in vivo. *British Journal of Clinical Pharmacology*, *80*, 493–501.

Bates, D. J. P., Salerni, B. L., Lowrey, C. H., & Eastman, A. (2011). Vinblastine sensitizes leukemia cells to cyclin-dependent kinase inhibitors, inducing acute cell cycle phase-independent apoptosis. *Cancer Biology and Therapy*, *12*, 314-325.

Bauvois, B., Dumont, J., Mathiot, C., & Kolb, J.-P. (2002). Production of matrix metalloproteinase-9 in early stage B-CLL: suppression by interferons. *Leukemia*, *16*, 791–798.

Beardmore, V. A., Hinton, H. J., Eftychi, C., Apostolaki, M., Armaka, M., Darragh, J., … Arthur, C. (2005). Generation and Characterization of p38β (MAPK11) Gene-Targeted Mice. *Molecular and Cellular Biology*, *25*(23), 10454–10464.

Beatty, G. L., & Gladney, W. L. (2015). Immune escape mechanisms as a guide for cancer immunotherapy. *Clinical Cancer Research*, *21*, 687–692.

Bébien, M., Salinas, S., Becamel, C., Richard, V., Linares, L., & Hipskind, R. a. (2003). Immediate-early gene induction by the stresses anisomycin and arsenite in human osteosarcoma cells involves MAPK cascade signaling to Elk-1, CREB and SRF. *Oncogene*, *22*(12), 1836–1847.

Beere, H. M. (2004). “The stress of dying”: the role of heat shock proteins in the regulation of apoptosis. *Journal of Cell Science*, *117*, 2641–2651.

Beere, H. M. (2005). Death versus survival: functional interaction between the apoptotic and stress-inducible heat shock protein pathways. *Journal of Clinical Investigations*, *10*, 2633–2639.

Bendall, L. J., Baraz, R., Juarez, J., Shen, W., & Bradstock, K. F. (2005). Defective p38 Mitogen-Activated Protein Kinase Signaling Impairs Chemotaxic but not Proliferative Responses to Stromal-Derived Factor-1A in Acute Lymphoblastic Leukemia. *Cancer Research*, *65*, 3290–3298.

Bennett, B. L., Sasaki, D. T., Murray, B. W., O’Leary, E. C., Sakata, S. T., Xu, W., … Anderson, D. W. (2001). SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. *Proceedings of the National Academy of Sciences of the United States of America*, *98*, 13681–13686.

Bennour, A., Ouahchi, I., Elloumi, M., Khelif, A., Saad, A., & Sennana, H. (2012). Comprehensivve analysis of BCR/ABL variants in chronic myeloid

leukemia patients using multiplex RT-PCR. *Clinical Laboratory*, *58*, 433–439.

Berglund, C. M. D., Radesäter, A. C., Persson, M. A. A., & Budd Haeberlein, S. L. (2004). UV-induced apoptosis in SH-SY5Y cells: Contribution to apoptosis by JNK signaling and cytochrome c. *Journal of Neuroscience Research*, *78*, 580-589.

Bhat, M., Robichaud, N., Hulea, L., Sonenberg, N., Pelletier, J., & Topisirovic, I. (2015). Targeting the translation machinery in cancer. *Nature Reviews*, *14*, 261–278.

Bio-Rad. (2017). Precision Plus Protein WesternC Standards. Retrieved from http://www.bio-rad.com/en-ch/sku/1610376-precision-plus-protein-westernc-blotting-standards

Blombery, P. A., Wong, S. Q., Hewitt, C. A., Dobrovic, A., Maxwell, E. L., Juneja, S., … Westerman, D. A. (2012). Detection of BRAF mutations in patients with hairy cell leukemia and related lymphoproliferative disorders. *Haematologica, 95,* 780-783.

Bode, A. M., & Dong, Z. (2007). The Functional Contrariety of JNK. *Molecular Carcinogenesis*, *46*, 591–598.

Bogoyevitch, M. A., Boehm, I., Oakley, A., Ketterman, A. J., & Barr, R. K. (2004). Targeting the JNK MAPK cascade for inhibition: Basic science and therapeutic potential. In *Biochimica et Biophysica Acta, 1697,* 89-101.

Bogoyevitch, M. A. (2005). Therapeutic promise of JNK ATP-noncompetitive inhibitors. *Trends in Molecular Medicine*, *11*, 232–239.

Bogoyevitch, M. A. (2006). The isoform-specific functions of the c-Jun N-terminal kinases (JNKs): Differences revealed by gene targeting. *BioEssays, 28,* 923-934.

Bogoyevitch, M. A., & Arthur, P. G. (2008). Inhibitors of c-Jun N-terminal kinases-JuNK no more? *Biochimica et Biophysica Acta - Proteins and Proteomics*, *1784*, 76–93.

Bogoyevitch, M. A., Gillespie-Brown, J., Ketterman, A. J., Fuller, S. J., Ben-Levy, R., Ashworth, A., … Sugden, P. (1996). Stimulation of the Stress-Activated Mitogen-Activated Protein Kinase Subfamilies in Perfused Heart. *Circulation Research*, *79*, 162–173.

Bogoyevitch, M. A., & Kobe, B. (2006). Uses for JNK: the Many and Varied Substrates of the c-Jun N-Terminal Kinases. *Microbiology and Molecular Cell Biology*, *70*, 1061–1095.

Bonny, C., Oberson, A., Negri, S., Sauser, C., & Schorderet, D. F. (2001). Cell-Permeable Peptide Inhibitors of JNK Novel Blockers of β -Cell Death. *Diabetes*, *50*, 77–82.

Bos, J. L. (1989). ras Oncogenes in Human Cancer: A Review. *Cancer Research*, *49*, 4682–4689.

Boulton, T. G., Nye, S. H., Robbins, D. J., Ip, N. Y., Radzlejewska, E., Morgenbesser, S. D., … Yancopoulos, G. . D. (1991). ERKs: A family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. *Cell*, *65*, 663–675.

Boulton, T. G., Yancopoulos, G. D., Gregory, J. S., Slaughter, C., Moomaw, C., & Hsu, J. (1990). An insulin-stimulated protein kinase similar to yeast kinases involved in cell cycle control. *Science*, *249*, 64–67.

Bradstock, K. F., & Gottlieb, D. J. (1995). Interaction of acute leukemia cells with the bone marrow micro-environment: implications for control of minimal residual disease. *Leukemia & Lymphoma* , *18*, 1–16.

Bradstock, K. F., Link, E., Di Iuliom, J., Szer, J., Marlton, P., Wei, A. H., … Seymour, J. F. (2017). Idarubicin Dose Escalation for Adult Acute Myeloid Leukemia. *Journal of Clinical Oncology*, *35*, 1678–1685.

Browett, P. J., & Norton, J. D. (1989). Analysis of ras gene mutations and methylation state in human leukemias. *Oncogene*, *4*, 1029–1036.

Brown, A. P., Carlson, T. C. G., Loi, C., & Graziano, M. J. (2007). Pharmacodynamic and toxicokinetic evaluation of the novel MEK inhibitor, PD0325901, in the rat following oral and intravenous administration. *Cancer Chemother Pharmacol*, *59*, 671–679.

Brozovic, A., Fritz, G., Christmann, M., Zisowsky, J., Jaehde, U., Osmak, M., & Kaina, B. (2004). Long-term activation of SAPK/JNK, p38 kinase and fas-L expression by cisplatin is attenuated in human carcinoma cells that acquired drug resistance. *International Journal of Cancer*, *112*, 974–985.

Bryne, J. L., & Marshall, C. J. (1998). The Molecular Pathophysiology of Myeloif Leukaemias: Ras Revisited. *British Journal of Haematology*, *100*, 256–264.

Bubici, C., & Papa, S. (2014). JNK signalling in cancer: in need of new, smarter therapeutic targets. *British Journal of Pharmacology*, *171*, 24-37.

Budhraja, A., Gao, N., Zhang, Z., Son, Y.-O., Cheng, S., Wang, X., … Shi, X. (2012). Apigenin Induces Apoptosis in Human Leukemia Cells and Exhibits Anti-Leukemic Activity In Vivo. *Molecular Cancer Therapeutics*, *11*, 132–142.

Bulavin, D. V., Higashimoto, Y., Popoff, I. J., Gaarde, W. A., Basrur, V., Potapova, O., … Forance. A. J. (2001). Initiation of a G2/M checkpoint after ultraviolet radiation requires p38 kinase. *Nature*, *411*, 102–107.

Burgess, G. S., Williamson, E. A., Cripe, L. D., Litz-Jackson, S., Bhatt, J. A., Stanley, K., … Boswell, H. S. (1998). Regulation of the c-jun Gene in p210 BCR-ABL Transformed Cells Corresponds With Activity of JNK, the c-jun N-Terminal Kinase. *Blood*, *92*, 2450–2460.

Buscà, R., Pouysségur, J., & Lenormand, P. (2016). ERK1 and ERK2 Map Kinases: Specific Roles or Functional Redundancy? *Frontiers in Cell and Developmental Biology*, *4*, 1-23.

Buschmann, T., Potapova, O., Bar-Shira, A., Ivanov, V. N., Fuchs, S. Y., Henderson, S., … Ronai, E. (2001). Jun NH 2 -Terminal Kinase Phosphorylation of p53 on Thr-81 Is Important for p53 Stabilization and Transcriptional Activities in Response to Stress, *21*, 2743–2754.

Cadet, J., Ravanat, J. L., TavernaPorro, M., Menoni, H., & Angelov, D. (2012). Oxidatively generated complex DNA damage: Tandem and clustered lesions. *Cancer Letters*, 327, 5-15.

Campbell, R. M., Anderson, B. D., Brooks, N. A., Brooks, H. B., Chan, E. M., De Dios, A., … Ye, X. S. (2014). Characterization of LY2228820 Dimesylate, a Potent and Selective Inhibitor of p38 MAPK with Antitumor Activity. *Molecular Cancer Therapeutics*, *13*, 364–374.

Cancer Research UK. (2017). Cancer Statistics for the UK. Retrieved from http://www.cancerresearchuk.org/health-professional/cancer-statistics

Cappellini, A., Tazzari, P. L., Mantovani, I., Billi, A. M., Tassi, C., Ricci, F., … Martelli, A. M. (2005). Antiapoptotic role of p38 mitogen activated protein kinase in Jurkat T cells and normal human T lymphocytes treated with 8-methoxysporlaen and ultraviolet-A radiation. *Apoptosis*, *10*, 141–152.

Carboni, S., Boschert, U., Gaillard, P., Gotteland, J.-P., Gillon, J.-Y., & Vitte, P.-A. (2008). AS601245, a c-Jun NH 2 -terminal kinase (JNK) inhibitor, reduces axon/dendrite damage and cognitive deficits after global cerebral ischaemia in gerbils. *British Journal of Pharmacology*, *153*, 157–163.

Carboni, S., Hiver, a., Szyndralewiz, C., Gaillard, P., Gotteland, J.-P., & Vitte, P.-A. (2004). AS601245 (1,3-Benzothiazol-2-yl (2-{[2-(3-pyridinyl) ethyl] amino}-4 pyrimidinyl) Acetonitrile): A c-Jun NH2-Terminal protein Kinase Inhibitor with Neuroprotective Properties. *The Journal of Pharmacology and Experimental Therapeutics*, *310*, 35–32.

Carella, A. M., Berman, E., Marone, M. P., & Ganzina, F. (1990). Idarubicin in the treatment of acute leukemias. An overview preclinical and clinical studies. *Haematologica*, *75*, 159–169.

Cargnello, M., & Roux, P. P. (2011). Activation and Function of the MAPKs and Their Substrates, the MAPK-Activated Protein Kinases. *Microbiology and Molecular Biology Reviews*, *75*, 50–83.

Catling, A. D., Schaeffer, H.-J., Reuter, C. W. M., Rukmini Reddy, G., & Weber, M. J. (1995). A Proline-Rich Sequence Unique to MEK1 and MEK2 Is Required for Raf Binding and Regulates MEK Function. *Molecular and Cell Biology*, *15*(10), 5214–5225.

Cavigelli, M., Dolfi, F., Claret, F., & Karin, M. (1995). Induction of c-fos expression through JNK-mediated TCF/Elk-1 phosphorylation. *The EMBO Journal*, *14*, 5957–5964.

Cerezo-Guisado, M. I., Zur, R., Lorenzo, M. J., Risco, A., Martín-Serrano, M. A., Alvarez-Barrientos, A., … Centeno, F. (2015). Implication of Akt, ERK1/2 and alternative p38MAPK signalling pathways in human colon

cancer cell apoptosis induced by green tea EGCG. *Food and Chemical Toxicology*, *84*, 125–132.

César Ortiz-Lazareno, P., Bravo-Cuellar, A., Lerma-Díaz, J. M., Jave-Suárez, L. F., Aguilar-Lemarroy, A., Ramiro Domínguez-Rodríguez, J., … Hernández-Flores, G. (2014). Sensitization of U937 leukemia cells to doxorubicin by the MG132 proteasome inhibitor induces an increase in apoptosis by suppressing NF-kappa B and mitochondrial membrane potential loss. *Cancer Cell International*, *14*, 1–14.

Chaikuad, A., Tacconi, E., Zimmer, J., Liang, Y., Gray, N. S., Tarsounas, M., & Knapp, S. (2014). A unique inhibitor binding site in ERK1/2 is associated with slow binding kinetics Europe PMC Funders Group. *Nat Chem Biol*, *10*, 853–860.

Chatterjee, D. K., Diagaradjane, P., & Krishnan, S. (2011). Nanoparticle-mediated hyperthermia in cancer therapy. *Therapeutic Delivery*, *2*, 1001–1014.

Chen, M., & Wang, J. (2002). Initiator caspases in apoptosis signalling pathways. *Apoptosis*, *7*, 313–319.

Chen, Y.-R., Wang, X., Templeton, D., Davis, R. J., & Tan, T.-H. (1996). The Role of c-Jun N-terminal Kinase (JNK) in Apoptosis Induced by Ultraviolet C and γ Radiation. *The Journal of Biological Chemistry*, *271*, 31929–31936.

Chen, Y., Zhao, Y., Wang, C., Xiao, X., Zhou, X., & Xu, G. (2012). Inhibition of p38 MAPK diminishes doxorubicin- induced drug resistance associated with P-glycoprotein in human leukemia K562 cells. *Medical Science Monitor*, *18*, 383–388.

Chen, Z., & Cobb, M. H. (2006). Activation of MEKK1 by Rho GTPases. *Methods in Enzymology*, *406*, 468–478.

Cheung-Ong, K., Giaever, G., & Corey, N. (2013). DNA-Damaging Agents in Cancer Chemotherapy: Serendipity and Chemical Biology. *Chemistry & Biology*, *20*, 648–659.

Cheung-Ong, K., Giaever, G., & Nislow, C. (2013). Chemistry & Biology Perspective DNA-Damaging Agents in Cancer Chemotherapy: Serendipity and Chemical Biology. *Chemistry & Biology*, *20*, 648–659.

Choo, E. F., Belvin, M., Boggs, J., Deng, Y., Hoeflich, K. P., Ly, J., … Johnston, S. H. (2012). Preclinical disposition of GDC-0973 and prospective and retrospective analysis of human dose and efficacy predictions. *Drug Metabolism and Disposition*, *40*, 919–927.

Chouinard, N., Valerie, K., Rouabhia, M., & Huot, J. (2002). UVB-mediated activation of p38 mitogen-activated protein kinase enhances resistance of normal human keratinocytes to apoptosis by stabilizing cytoplasmic p53. *Biochemical Journal*, *365*, 133–145.

Clerk, A., Fuller, S. J., Michael, A., & Sugden, P. H. (1998). Stimulation of

"Stress-regulated" Mitogen-activated Protein Kinases (Stress-activated Protein Kinases/c-Jun N-terminal Kinases and p38-Mitogen-activated Protein Kinases) in Perfused Rat Hearts by Oxidative and Other Stresses. *The Journal of Biological Chemistry*, *273*, 7228–7234.

Cohen, S. b., Cheng, T.-T., Chindalore, V., Damjanov, N., Burgos-Vargas, R., Delora, P., … Caulfield, J. P. (2009). Evaluation of the Efficacy and Safety of Pamapimod, a p38 MAP Kinase Inhibitor, in a Double-Blind, Methotrexate-Controlled Study of Patients With Active Rheumatoid Arthritis. *Arthritis & Rheumatism*, *60*, 335–344.

Colotta, F., Allavena, P., Sica, A., Garlanda, C., & Mantovani, A. (2009). Cancer-related inflammation, the seventh hallmark of cancer: Links to genetic instability. *Carcinogenesis*, *30*, 1073–1081.

Cortes, J. E., Kantarjian, H., Foran, J. M., Ghirdaladze, D., Zodelava, M., Borthakur, G., … Levis, M. (2013). Phase I study of quizartinib administered daily to patients with relapsed or refractory acute myeloid leukemia irrespective of FMS-like tyrosine kinase 3-internal tandem duplication status. *Journal of Clinical Oncology , 31,* 3681-3689.

Coulombe, P., & Meloche, S. (2007). Atypical mitogen-activated protein kinases: Structure, regulation and functions. *Biochimica et Biophysica Acta, 1773,* 1376-1387.

Coussens, L. M., & Werb, Z. (2002). Inflammation and cancer. *Natue*, *420*, 860–867.

Creagh, E. M., Sheehan, D., & Cotter, T. G. (2000). Heat shock proteins – modulators of apoptosis in tumour cells. *Leukemia*, *14*, 1161–1173.

Cripe, L. D., Gelfanov, V. M., Smith, E. A., Spigel, D. R., Phillips, C. A., Gabig, T. G., … Boswell, H. S. (2002). Role for c-jun N-terminal kinase in treatment-refractory acute myeloid leukemia (AML): signaling to multidrug-efflux and hyperproliferation. *Leukemia*, *16*, 799–812.

Cristóbal, I., Garcia-Orti, L., Cirauqui, C., Alonso, M., & Calasanz, M. (2011). PP2A impaired activity is a common event in acute myeloid leukemia and its activation by forskolin has a potent anti-leukemic effect. *Leukemia*, *25*, 606–614.

Cuadrado, A., & Nebreda, A. R. (2010). Mechanisms and functions of p38 MAPK signalling. *Biochem. J*, *429*, 403–417.

Cuda, C. M., Pope, R. M., & Perlman, H. (2016). The inflammatory role of phagocyte apoptotic pathways in rheumatic diseases. *Nature Reviews Rheumatology*, *12*, 543–558.

Cuenda, A., Rouse, J., Doza, Y. N., Meier, R., Cohen " ', P., Gallagher, T. F., … Lee, J. C. (1995). SB 203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. *FEBS Letters*, *364,* 229-233.

Cuenda, A., & Rousseau, S. (2007). p38 MAP-Kinases pathway regulation,

function and role in human diseases. *Biochimica et Biophysica Acta, 1773*, 1358-1375.

Cuevas, B., Abell, A., & Johnson, G. (2007). Role of mitogen-activated protein kinase kinase kinases in signal integration. *Oncogene*, *26*, 3159–3171.

Cui, J., Wang, Q., Wang, J., Lv, M., Zhu, N., Li, Y., … Zhang, J. (2009). Basal c-Jun NH2-terminal protein kinase activity is essential for survival and proliferation of T-cell acute lymphoblastic leukemia cells. *Molecular Cancer Therapeutics*, *8*, 3214–3222.

Cui, J., Zhang, M., Zhang, Y. Q., & Xu, Z. H. (2007). JNK pathway: Diseases and therapeutic potential. *Acta Pharmacologica Sinica*, *28*, 601–608.

Cuozzo, F., Raciti, M., Bertelli, L., Parente, R., & Di Renzo, L. (2012). Pro-death and pro-survival properties of ouabain in U937 lymphoma derived cells. *Journal of Experimental & Clinical Cancer Research*, *31*, 1-8.

Dai, Y., Dent, P., & Grant, S. (2003). Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand (TRAIL) Promotes Mitochondrial Dysfunction and Apoptosis Induced by 7-Hydroxystaurosporine and Mitogen-Activated Protein Kinase Kinase Inhibitors in Human Leukemia Cells That Express Bcl-2 and Bcl-xL. *Molecular Pharmacology*, *64*, 1402–1409.

Dai, Y., Yu, C., Singh, V., Tang, L., Wang, Z., Mcinistry, R., … Grant, S. (2001). Pharmacological Inhibitors of the Mitogen-activated Protein Kinase (MAPK) Kinase/MAPK Cascade Interact Synergistically with UCN-01 to Induce Mitochondrial Dysfunction and Apoptosis in Human Leukemia Cells 1. *Cancer Research*, *61*, 5106–5115.

Davies, B. R., Logie, A., Mckay, J. S., Martin, P., Steele, S., Jenkins, R., … Smith, P. D. (2007). AZD6244 (ARRY-142886), a potent inhibitor of mitogen- activated protein kinase/extracellular signal-regulated kinase kinase 1/2 kinases: mechanism of action in vivo, pharmacokinetic/pharmacodynamic relationship, and potential for combination in preclinical models. *Molecular Cancer Therapeutics*, *6*, 2209–19.

Davies, S. P., Reddy, H., Caivano, M., & Cohen, P. (2000). Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem. J*, *351*, 95–105.

Davison, K., Mann, K. K., Waxman, S., & Miller, W. H. (2004). JNK activation is a mediator of arsenic trioxide–induced apoptosis in acute promyelocytic leukemia cells. *Blood*, *103*, 3496–3502.

Deak, M., Clifton, A. D., Lucocq, J. M., & Alessi, D. R. (1998). Mitogen- and stress-activated protein kinase-1 (MSK1) is directly activated by MAPK and SAPK2/p38, and may mediate activation of CREB. *EMBO Journal*, *17*, 4426–41.

Demidenko, Z., Vivo, C., Halicka, H., Li, C., Bhalla, K., Broude, E., & Blagosklonny, M. (2006). Pharmacological induction of Hsp70 protects

apoptosis-prone cells from doxorubicin: comparison with caspase-inhibitor-and cycle-arrest-mediated cytoprotection. *Cell Death and Differentiation*, *13*, 1434–1441.

Dempsey, N. C. (2009). *Localisation of heat shock proteins in haematological malignanices*. University of Liverpool.

DeNardo, G. L., & DeNardo, S. J. (2008). Turning the Heat on Cancer. *Cancer Biotherapy & Radiopharmaceuticals*, *23*, 671–678.

Derijard, B., Hibi, M., Wu, I. H., Barrett, T., Su, B., Deng, T., … Davis, R. J. (1994). JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. *Cell*, *76*, 1025–1037.

Derijard, B., Raingeaud, J., Barrett, T., Wu, I. H., Ulevitch, R. J., & Davis, R. J. (1995). Independent human MAP-kinase signal transduction pathways defined by MEK and MKK isoforms. *Science*, *267*, 682–685.

Dhanasekaran, D., & Johnson, G. (2007). MAPKs: function, regulation, role in cancer and therapeutic targeting. *Oncogene*, *26*, 3097–3099.

Dickens, M., Rogers, J. S., Cavanagh, J., Raitano, A., Xia, Z., Halpern, J. R., … Davis, R. J. (1997). A Cytoplasmic Inhibitor of the JNK Signal Transduction Pathway. *Science*, *277*, 693–696.

Dickinson, R. J., & Keyse, S. M. (2006). Diverse physiological functions for dual-specificity MAP kinase phosphatase. *Journal of Cell Science*, *119*, 4607–4615.

Dietrich, S., Pircher, A., Endris, V., eric Peyrade, F., Wendtner, C.-M., Follows, G. A., … Zenz, T. (2016). BRAF inhibition in hairy cell leukemia with low-dose vemurafenib. *Blood*, *127*, 2847–2855.

Doménech, E., Gómez-López, G., Gzlez-Peña, D., López, M., Herreros, B., Menezes, J., … Sánchez-Beato, M. (2012). New mutations in chronic lymphocytic leukemia identified by target enrichment and deep sequencing. *PLoS ONE, 7,* 1-6 .

Dorion, S., Bérubé, J., Huot, J., & Landry, J. (1999). A short lived protein involved in the heat shock sensing mechanism responsible for stress-activated protein kinase 2 (SAPK2/p38) activation. *Journal of Biological Chemistry*, *274*, 37591–37597.

Dorion, S., Lambert, H., & Landry, J. (2002). Activation of the p38 signaling pathway by heat shock involves the dissociation of glutathione S-transferase Mu from Ask1. *Journal of Biological Chemistry*, *277*, 30792–30797.

Dudley, D. T., Pang, L., Decker, S. J., Bridgest, A. J., & Saltiel, A. R. (1995). A synthetic inhibitor of the mitogen-activated protein kinase cascade (protein phosphorylation/growth factor/cell proliferation). *Biochemistry*, *92*, 7686–7689.

Duffy, J. P., Harrington, E. M., Salituro, G., Cochran, J. E., Green, J., Gao, H., … Su, M. S. S. (2011). The Discovery of VX-745: A Novel and Selective p38α Kinase Inhibitor. *ACS Medicinal Chemistry Letters*, *2*, 758–763.

Dumka, D., Puri, P., Carayol, N., Lumby, C., Balachandran, H., Schuster, K., … Parmar, S. (2009). Activation of the p38 Map Kinase Pathway is Essential for the Antileukemic Effects of Dasatinib. *Leukemia & Lymphoma* , *50*, 2017–2029.

Dumontet, C., & Jordan, M. (2010). Microtubule-binding agents: a dynamic field of cancer therapeutics. *Nature Reviews. Drug Discovery*, *9*, 790–803.

Duncia, J. V., Santella, J. B., Higley, C. A., Pitts, W. J., Wityak, J., Frietze, W. E., … Olson, R. E. (1998). MEK inhibitors: The chemistry and biological activity of U0126, its analogs, and cyclization products. *Bioorganic and Medicinal Chemistry Letters*, *8*, 2839–2844.

Ebelt, N. D., & van Den Berg, C. L. (2013). The irreversible c-Jun N-terminal kinase (JNK) inhbitor, JNK-IIN-9, sensitizes basal-like breast cancer cells to lapatinib. *Cancer Research*, *73*, 24.

Elmore, S. (2007). Apoptosis: A Review of Programmed Cell Death. *Toxicologic Pathology*, *35*, 495–516.

Elsea, C. R., Roberts, D. A., Druker, B. J., & Wood, L. J. (2008). Inhibition of p38 MAPK suppresses inflammatory cytokine induction by etoposide, 5-fluorouracil, and doxorubicin without affecting tumoricidal activity. *PLoS ONE*, *3*, 2355–2363.

Enslen, H., Raingeaud, J. L., & Davis, R. J. (1998). Selective Activation of p38 Mitogen-activated Protein (MAP) Kinase Isoforms by the MAP Kinase Kinases MKK3 and MKK6. *The Journal of Biological Chemistry*, *273*, 1741–1748.

Eriksson, M., Taskinen, M., & Leppä, S. (2006). Mitogen Activated Protein Kinase-Dependent Activation of c-Jun and c-Fos is required for Neuronal differentiation but not for Growth and Stress Reposne in PC12 cells. *Journal of Cellular Physiology*, *207*, 12–22.

Escós, A., Risco, A., Alsina-Beauchamp, D., & Cuenda, A. (2016). p38γ and p38δ Mitogen Activated Protein Kinases (MAPKs), New Stars in the MAPK Galaxy. *Frontiers in Cell and Developmental Biology*, *4*, 1–7.

Falchook, G. S., Long, G. V., Kurzrock, R., Kim, K. B., Arkenau, T. H., Brown, M. P., … Kefford, R. F. (2012). Dabrafenib in patients with melanoma, untreated brain metastases, and other solid tumours: A phase 1 dose-escalation trial. *The Lancet*, *379*, 1893–1901.

Falini, B., Martelli, M. P., & Tiacci, E. (2016). BRAF V600E mutation in hairy cell leukemia: from bench to bedside. *Blood*, *128*, 1918–1927.

Falk, M. H., & Issels, R. D. (2001). Hyperthermia in oncology. *International Journal of Hyperthermia*, *17*, 1–18.

Fallahi-Sichani, M., Moerke, N. J., Niepel, M., Zhang, T., Gray, N. S., & Sorger, P. K. (2015). Systematic analysis of BRAFV600E melanomas reveals a role for JNK/c-Jun pathway in adaptive resistance to drug-induced apoptosis. *Molecular Systems Biology*, *11*, 1-1.

Fan, M., & Chambers, T. C. (2001). Role of mitogen-activated protein kinases in the response of tumor cells to chemotherapy. *Drug Resistance Updates*, *5*, 253–267.

Fanger, G. R., Gerwins, P., Widmann, C., Jarpe, M. B., & Johnson, G. L. (1997). MEKKs, GCKs, MLKs, PAKs, TAKs and Tpls: upstream regulators of the c-Jun amino-terminal kinases? *Current Opinion in Genetics & Development*, *7*, 67–74.

Farr, C. J., Saikit, R. K., Erlicht, H. A., Mccormickf, F., & Marshall, C. J. (1988). Analysis of RAS gene mutations in acute myeloid leukemia by polymerase chain reaction and oligonucleotide probes. *Medical Sciences*, *85*, 1629–1633.

Fathi, A. T., & Chen, Y. (2011). Treatment of FLT3-ITD acute myeloid leukemia. *American Journal of Blood Research*, *1*, 175–189.

Favata, M. F., Horiuchi, K. Y., Manos, E. J., Daulerio, A. J., Stradley, D. A., Feeser, W. S., … Trzaskos, J. M. (1998). Identification of a Novel Inhibitor of Mitogen-activated Protein Kinase Kinase. *The Journal of Biological Chemistry*, *273*, 18623–18632.

Feng, Y., Wen, J., & Chang, C.-C. (2009). p38 Mitogen-Activated Protein Kinase and Hematologic Malignancies. *Archives of Pathology & Laboratory Medicine*, *133*, 1850–1856.

Fernald, K., & Kurokawa, M. (2013). Evading apoptosis in cancer. *Trends in Cell Biology*, *23*, 620–633.

Festjens, N., Berghe, T. V., & Vandenabeele, P. (2006). Necrosis, a well-orchestrated form of cell demise: Signalling cascades, important mediators and concomitant immune response. *Biochimica et Biophysica Acta*, *1757*, 1371–1387.

Fischer, T., Stone, R. M., DeAngelo, D. J., Galinsky, I., Estey, E., Lanza, C., … Giles, F. J. (2010). Phase IIB trial of oral midostaurin (PKC412), the FMS-like tyrosine kinase 3 receptor (FLT3) and multi-targeted kinase inhibitor, in patients with acute myeloid leukemia and high-risk myelodysplastic syndrome with either wild-type or mutated FLT3. *Journal of Clinical Oncology, 28,* 4339-4345.

Flaherty, K. T., Puzanov, I., Kim, K. B., Ribas, A., McArthur, G. A., Sosman, J. A., … Chapman, P. B. (2010). Inhibition of Mutated, Activated BRAF in Metastatic Melanoma. *New England Journal of Medicine*, *363*, 809–819.

Fox, T., Coll, J. T., Xie, X., Ford, P. J., Germann, U. A., Porter, M. D., … Wilson, K. P. (1998). A single amino acid substitution makes ERK2 susceptible to pyridinyl imidazole inhibitors of p38 MAP kinase. *Protein Science, 7*,

72249–2255.

Franklin, C. C., Srikanth, S., Kraft, A. S., & Prescott, D. M. (1998). Conditional expression of mitogen-activated protein kinase phosphatase-1, MKP-1, is cytoprotective against UV-induced apoptosis. *Cell Biology*, *95*, 3014–3019.

Frémin, C., & Meloche, S. (2010). From basic research to clinical development of MEK1/2 inhibitors for cancer therapy. *Journal of Hematology, 3*, 2-11.

Freshney, N., Rawlinson, L., Guesdon, F., Jones, E., Cowley, S., Hsuan, J., & Saklatvala, J. (1994). Interleukin-1 activates a novel protein kinase cascade that results in the phosphorylation of hsp27. *Cell*, *78*, 1039–1049.

Fridman, J. S., & Lowe, S. W. (2003). Control of apoptosis by p53. *Oncogene*, *22*, 9030–9040.

Fu, J., Meng, X., He, J., & Gu, J. (2008). Inhibition of Inflammation by a p38 MAP Kinase Targeted Cell Permeable Peptide. *Medicinal Chemistry*, *4*, 597–604.

Fukuda, M., Gotoh, I., Gotoh, Y., & Nishida, E. (1996). Cytoplasmic Localization of Mitogen-activated Protein Kinase Kinase Directed by Its NH 2 -terminal, Leucine-rich Short Amino Acid Sequence, Which Acts as a Nuclear Export Signal. *The Journal of Biological Chemistry*, *271*, 20024–20028.

Fukunaga, R., & Hunter, T. (1997). MNK1, a new MAP kinase-activated protein kinase, isolated by a novel expression screening method for identifying protein kinase substrates. *EMBO Journal*, *16*, 1921–1933.

Fulda, S., & Debatin, K.-M. (2006). Extrinsic versus intrinsic apoptosis pathways in anticancer chemotherapy. *Oncogene*, *25*, 4798–4811.

Fulda, S., Gorman, A. M., Hori, O., & Samali, A. (2010). Cellular stress responses: Cell survival and cell death. *International Journal of Cell Biology, 201,* 1-23.

Gabai, V. L., Meriin, A. B., Mosser, B. D., Caron, A. W., Rits, S., Shifrin, V. I., & Sherman, M. Y. (1997). Hsp70 prevents activation of stress kinases: A novel pathway of cellular thermotolerance. *Journal of Biological Chemistry*, *272*, 18033–18037.

Gaillard, P., Jeancalude-Etter, I., Ardissone, V., Arkinstall, S., Cambet, Y., Camps, M., … Gotteland, J.-P. (2005). Design and Synthesis of the First Generation of Novel Potent, Selective, and in Vivo Active (Benzothiazol-2-yl)acetonitrile Inhibitors of the c-Jun N-Terminal Kinase. *Journal of Medicinal Chemistry*, *48*, 4596–4607.

Gallagher, E. D., Gutowski, S., Sternweis, P. C., & Cobb, M. H. (2004). RhoA Binds to the Amino Terminus of MEKK1 and Regulates Its Kinase Activity. *Journal of Biological Chemistry*, *279*, 1872–1877.

Gallo, K. A., & Johnson, G. L. (2002). Mixed-lineage kinase control of JNK and

p38 MAPK pathways. *Nature Reviews Molecular Cell Biology*, *3*, 663–673.

Gilliland, D., & Griffin, J. D. (2002). The roles of FLT3 in hematopoiesis and leukemia. *Blood, 100,* 1532-1542.

Gaundar, S. S., Bradstock, K. F., & Bendall, L. J. (2009). p38MAPK inhibitors attenuate cytokine production by bone marrow stromal cells and reduce stroma-mediated proliferation of acute lymphoblastic leukemia cells. *Cell Cycle*, *8*, 2977–2985.

Giafis, N., Katsoulidis, E., Sassano, A., Tallman, M. S., Higgins, L. S., Nebreda, A. R., … Platanias, L. C. (2006). Role of the p38 mitogen-activated protein kinase pathway in the generation of arsenic trioxide-dependent cellular responses. *Cancer Research*, *66*, 6763–6771.

Giancotti, F. G. (2014). Deregulation of cell signaling in cancer. *FEBS Letters, 588,* 2558-2570.

Giles, F., & Estey, E. (2003). Gemtuzumab Ozogamicin in the Treatment of Acute Myeloid Leukemia. *Cancer*, *98*, 2095–2104.

Gill, K., Nigam, L., Singh, R., Kumar, S., Subbarao, N., Chauhan, S. S., & Dey, S. (2014). The Rational Design of Specific Peptide Inhibitor against p38α MAPK at Allosteric-Site: A Therapeutic Modality for HNSCC. *PLoS ONE*, *9*, 1–10.

Gill, K., Singh, A. K., Kapoor, V., Nigam, L., Kumar, R., Holla, P., … Dey, S. (2013). Development of peptide inhibitor as a therapeutic agent against head and neck squamous cell carcinoma (HNSCC) targeting p38α MAP kinase. *Biochimica et Biophysica Acta*, *1830*, 2763–2769.

Gilmartin, A. G., Bleam, M. R., Groy, A., Moss, K. G., Minthorn, E. A., Kulkarni, S. G., … Laquerre, S. G. (2011). GSK1120212 (JTP-74057) is an inhibitor of MEK activity and activation with favorable pharmacokinetic properties for sustained in vivo pathway inhibition. *Clinical Cancer Research*, *17*, 989–1000.

Giustini, A. J., Petryk, A. A., Cassim, S. M., Tate, J. A., Baker, I., & Hoopes, P. J. (2010). Magnetic nanoparticle hyperthermia in cancer treatment. *Nano Life*, *1*, 1–23.

Gong, Q., Zhou, L., Xu, S., Li, X., Zou, Y., & Chen, J. (2015). High doses of daunorubicin during induction therapy of newly diagnosed acute myeloid leukemia: A systematic review and meta-analysis of prospective clinical trials. *PLoS ONE*, *10*, 1–14.

Gotoh, Y., & Nishida, E. (1995). Activation Mechanism and Function of the MAP Kinase Cascade. *Molecular Reproduction and Development*, *42*, 486–492.

Grollman, A. P. (1967). Inhibitors of Protein Biosynthesis II. Mode of Action of Anisomycin. *The Journal of Biological Chemistry*, *242*, 3226–3233.

Grumbach, I. M., Mayer, I. A., Uddin, S., Lekmine, F., Majchrzak, B., Yamauchi, H., … Platanias, L. C. (2001). Engagement of the CrkL adaptor in interferon a signalling in BCR±ABL-expressing cells. *British Journal of Haematology*, *112*, 327–336.

Guicciardi, M. E., & Gores, G. J. (2009). Life and death by death receptors. *The FASEB Journal*, *23*, 1625–1637.

Gupta, M., Gupta, S. K., Hoffman, B., & Liebermann, D. A. (2006). Gadd45a and Gadd45b protect hematopoietic cells from UV-induced apoptosis via distinct signaling pathways, including p38 activation and JNK inhibition. *The Journal of Biological Chemistry*, *281*, 17552–17558.

Gupta, S., Campbell, D., Derijard, B., & Davis, R. J. (1995). Transcription factor ATF2 regulation by the JNK signal transduction pathway. *Science*, *267*, 389–393.

Gururajan, M., Chui, R., Karuppannan, A., Ke, J., Jennings, D., & Bondada, S. (2005). c-Jun N-terminal kinase (JNK) is required for survival and proliferation of B-lymphoma cells. *Blood*, *106*, 1382–1391.

Gutierrez, A., Pan, L., Groen, R. W. J., Baleydier, F., Kentsis, A., Marineau, J., … Aster, J. C. (2014). Phenothiazines induce PP2A-mediated apoptosis in T cell acute lymphoblastic leukemia. *Journal of Clinical Investigation 124,* 644-655.

Gutschner, T., & Diederichs, S. (2012). The hallmarks of cancer: A long non-coding RNA point of view. *RNA Biology 703 RNA Biology*, *9*, 703–719.

Haddad, J. J. (2001). VX-745 Vertex Pharmaceuticals. *Current Opinion in Investigational Drugs*, *2*, 1070–1076.

Haeusgen, W., Herdegen, T., & Waetzig, V. (2011). The bottleneck of JNK signaling: Molecular and functional characteristics of MKK4 and MKK7. *European Journal of Cell Biology*, *90*, 536–544.

Halaban, R., Zhang, W., Bacchiocchi, A., Cheng, E., Parisi, F., Ariyan, S., … Sznol, M. (2010). PLX4032, a selective BRAF V600E kinase inhibitor, activates the ERK pathway and enhances cell migration and proliferation of BRAF WT melanoma cells. *Pigment Cell Melanoma Research*, *23*, 190–200.

Hallak, M., Win, T., Shpilberg, O., Bittner, S., Granot, Y., Levy, I., & Nathan, I. (2009). The anti-leukaemic activity of novel synthetic naphthoquinones against acute myeloid leukaemia: Induction of cell death via the triggering of multiple signalling pathways. *British Journal of Haematology*, *147*, 459–470.

Han, J., Lee, J. D., Bibbs, L., & Ulevitch, R. J. (1994). A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. *Science*, *265*, 808–811.

Hanada, M., Noguchi, T., & Yamaoka, T. (2006). Amrubicin induces apoptosis in human tumor cells mediated by the activation of caspase-3/7 preceding

a loss of mitochondrial membrane potential. *Cancer Science, 97,* 1396-1403.

Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *Cell*, *144*, 646–674.

Hanahan, D., Weinberg, R. A. (2000). The hallmarks of cancer. *Cell*, *100*, 57–70.

Hatzivassilou, G., Song, K., Yen, I., Brandhuber, B. J., Anderson, D. J., Alvarado, R., … Malek, S. (2010). RAF inhibitors prime wild-type RAF to activate the MAPK pathway and enhance growth. *Nature*, *464*, 431–436.

Hauschild, A., Grob, J., Demidov, L. V., Jouary, T., Gutzmer, R., MIllward, M., … Chapman, P. B. (2012). Dabrafenib in BRAF-mutated metastatic melanoma: a multicentre, open-lable, phae 3 randomised controlled trial. *The Lancet*, *380*, 358–365.

Hazlehurst, L. A., Valkov, N., Wisner, L., Storey, J. A., Boulware, D., Sullivan, D. M., & Dalton, W. S. (2001). Reduction in drug-induced DNA double-strand breaks associated with β1 integrin-mediated adhesion correlates with drug resistance in U937 cells. *Blood, 98,* 1897-1903.

Hazzalin, C. A., Cano, E., Cuenda, A., Barratt, M. J., Cohen, P., & Mahadevan, L. C. (1996). p38/RK is essential for stress-induced nuclear responses: JNK/SAPKs and c-Jun/ATF-2 phosphorylation are iinsufficient. *Current Biology*, *6*, 1028–1031.

Heo, Y-S., Kim, S.-K., Seo, C., Kim, Y. K., Sung, B-J., Lee, H. S., … Yang, C-H. (2004). Structural basis for the selective inhibition of JNK1 by the scaffolding protein JIP1 and SP600125. *The EMBO Journal*, *23*, 2185–2195.

Hess, P., Pihan, G., Sawyers, C. L., Favell, R. A., & Davis, R. J. (2002). Survival signalling mediated by c-Jun NH(2)-terminal kinase in transformed B lymphoblasts. *Nature Genetics*, *32*, 201–205.

Hideshima, T., Hayashi, T., Chauhan, D., Akiyama, M., Richardson, P., & Anderson, K. (2003). Biologic sequelae of c-Jun NH(2)-terminal kinase (JNK) activation in multiple myeloma cell lines. *Oncogene*, *22*, 8797–8801.

Hildebrandt, B., Wust, P., Ahlers, O., Dieing, A., Sreenivasa, G., Kerner, T., … Riess, H. (2002). The cellular and molecular basis of hyperthermia. *Critical Reviews in Oncology*, *43*, 33–56.

Hill, R. J., Dabbagh, K., Phippard, D., Li, C., Suttmann, R. T., Welch, M., … Wong, B. R. (2008). Pamapimod, a Novel p38 Mitogen-Activated Protein Kinase Inhibitor: Preclinical Analysis of Efficacy and Selectivity. *Journal of Pharmacology and Experimental Therapeutics*, *327*, 610–619.

Hisatsune, J., Nakayama, M., Isomoto, H., Kurazono, H., Mukaida, N., Mukhopadhyay, A., … Hirayama. (2008). Molecular Characterisation of Helicobacter pylori VacA Induction of IL-8 in U937 Cells Reveals a

Prominent Role for p38MAPK in Activating Transcription Factor-2, cAMP Response Element Binding Protein, and NF-kB Activation. *The Journal of Immunology*, *180*, 5017–5027.

Holderfield, M., Deuker, M. M., Mccormick, F., & Mcmahon, M. (2014). Targeting RAF kinases for cancer therapy: BRAF mutated melanoma and beyond. *Nature Reviews Cancer*, *14*, 455–467.

Holzberg, D., Knight, C. G., Dittrich-Breiholz, O., Schneider, H., Dörrie, A., Hoffmann, E., … Kracht, M. (2003). Disruption of the c-JUN-JNK complex by a cell-permeable peptide containing the c-JUN δ domain induces apoptosis and affects a distinct set of interleukin-1-induced inflammatory genes. *The Journal of Biological Chemistry*, *278*, 40213–40223.

Hori, T., Kondo, T., Tabuchi, Y., Takasaki, I., Zhao, Q. L., Kanamori, M., … Kimura, T. (2008). Molecular mechanism of apoptosis and gene expressions in human lymphoma U937 cells treated with anisomycin. *Chemico-Biological Interactions, 172,* 125-140.

Hsiao, P.-C., Chou, Y. E., Tan, P., Lee, W. J., Yang, S. F., Chow, J. M., … Chien, M. H. (2014). Pterostilbene simultaneously induced G0/G1-phase arrest and MAPK-mediated mitochondrial-derived apoptosis in human acute myeloid leukemia cell lines. *PLoS ONE*, *9*, 105342–105356.

Huh, J., Kang, K., Chae, C., & Kim, H. (2004). Roles of p38 and JNK mitogen-activated protein kinase pathways during cantharidin-induced apoptosis in U937 cells, *67*, 1811–1818.

Huschtscha, L., Bartierl, W., Ross, A., & Tattersall, M. (1996). Characteristics of cancer cell death after exposure to cytotoxic drugs in vitro. *British Journal of Cancer*, *73*, 54–60.

Ichijo, H., Nishida, E., Irie, K., Dijke, P., Saitoh, M., Moriguchi, T., … Gotoh, Y. (1997). Induction of Apoptosis by ASK1, a Mammalian MAPKKK That Activates SAPK/JNK and p38 Signaling Pathways. *Science*, *275*, 90–94.

Irving, J., Matheson, E., Minto, L., Blair, H., Case, M., Halsey, C., … Eckert, C. (2014). Ras pathway mutations are prevalent in relapsed childhood acute lymphoblastic leukemia and confer sensitivity to MEK inhibition. *Blood*, *124*, 3420–3430.

Ivanov, V. N., & Ronai, Z. (2000). p38 protects human melanoma cells from UV-induced apoptosis through down-regulation of NF-kB activity and Fas expression. *Oncogene*, *19*, 30003–3012.

Jackson, S. P., & Bartek, J. (2009). The DNA-damage response in human biology and disease. *Nature*, *461*, 1071–1078.

Jacobs-Helber, S. M., & Sawyer, S. T. (2004). Jun N-terminal kinase promotes proliferation of immature erythroid cells and erythropoietin-dependent cell lines. *Blood*, *104*, 696–703.

Janssen, J. W. G., Steenvoorden, A. C. M., Lyons, J., Angert, B., Bohlket, J. U., Bost, J. L., … Bartram, C. R. (1987). RAS gene mutations in acute and

chronic myelocytic leukemias, chronic myeloproliferative disorders, and myelodysplastic syndromes. *Medical Sciences*, *84*, 9228–9232.

Jian Hua, Z. (2000). DNA fragmentation in apoptosis. *Cell Research*, *10*, 205–211.

Jiang, W. G., Sanders, A. J., Katoh, M., Ungefroren, H., Gieseler, F., Prince, M., … Santini, D. (2015). Tissue invasion and metastasis: Molecular, biological and clinical perspectives. *Seminars in Cancer Biology*, *35*, 244–275.

Jiang, Y., Chen, C., Li, Z., Guo, W., Gegner, J. A., Lin, S., & Han, J. (1996). Characterization of the Structure and Function of a New Mitogen-activated Protein Kinase (p38β). *The Journal of Biological Chemistry*, *271*, 17920–17926.

Johnson, G. L., Dohlman, H. G., & Graves, L. M. (2005). MAPK kinase kinases (MKKKs) as a target class for small-molecule inhibition to modulate signaling networks and gene expression. *Current Opinion in Chemical Biology, 9,* 325-331.

Johnson, G. L., & Nakamura, K. (2007). The c-Jun Kinase/Stress-activated Pathway: Regulation, Function and Role in Human Disease. *Biochimica et Biophysica Acta*, *1773*, 1341–1348.

Jolly, C., & Morimoto, R. I. (2000). Role of the Heat Shock Response and Molecular Chaperones in Oncogenesis and Cell Death. *Journal of the National Cancer Institute*, *92*, 1564–1572.

Jordan, M., & Wilson, L. (2004). Microtubules as a target for anticancer drugs. *Nature Reviews Cancer*, *4*, 253–265.

Juarez, J. G., Thien, M., Dela Pena, A., Baraz, R., Bradstock, K. F., & Bendall, L. J. (2009). CXCR4 mediates the homing of B cell progenitor acute lymphoblastic leukaemia cells to the bone marrow via activation of p38MAPK. *British Journal of Haematology, 145,* 491-499.

Kanagal-Shamanna, R., Luthra, R., Luthra, M., Lu, X., Routbort, M., Jain, N., … Patelm K. P. (2015). RAS/RAF Pathway Mutations Define a Distinct Subset of Chronic Lymphocytic Leukemia. *Blood*, *126*, 1730–1731.

Kaneko, M., Saito, Y., Saito, H., Matsumoto, T., Matsuda, Y., Vaught, J. L., … Murakata, C. (1997). Neurotophic 3,9-bis[(alkylthio)methyl]-and-bis(alkoxymethyll)-K-252a derviatives. *Jouranl of Medicinal Chemistry*, *40*, 1863–1869.

Kaspers, G. J. L., Pieters, R., Van Zantwijk, C. H., De Laat, P., De Waal, F., Van Wering, E., & Veerman, A. (1991). In vitro drug sensitivity of normal peripheral blood lymphocytes and childhood leukaemic cells from bone marrow and peripheral blood. *Brisiish Journal of Cancer* , *19*, 6–74.

Kato, T., Noma, H., Kitagawa, M., Takahashi, T., Oshitani, N., & Kitagawa, S. (2008). Distinct role of c-Jun N-terminal kinase isoforms in human neutrophil apoptosis regulated by tumour necrosis factor-alpha and

granulocyte-macrophage colony-stimulatiing factor. *Journal of Interfon and Cytokine Research*, *28*, 235–243.

Kennedy, D., Jäger, R., Mosser, D. D., & Samali, A. (2014). Regulation of apoptosis by heat shock proteins. *International Union of Biochemistry and Molecular Biology*, *66*, 327–338.

Kerr, A. H. J., James, J. A., Smith, M. A., Willson, C., Court, E. L., & Smith, J. G. (2003). An Investigation of the MEK/ERK Inhibitor U0126 in Acute Myeloid Leukemia. *Annals of the New York Academy of Sciences*, *1010*, 86–89.

Kerr, J. F. R., Wyllie, A. H., & Curriet, A. R. (1972). Apoptosis: A basic biological phenomenon with wide-ranging implications in tissue kinetics. *British Journal of Cancer*, *26*, 239–257.

Khan, K. H., Yap, T. A., Yan, L., & Cunningham, D. (2013). Targeting the PI3K-AKT-mTOR singnaling network in cancer. *Chinese Journal of Cancer*, *32*, 253–265.

Kiburg, B., Van De Loosdrecht3, A. A., Schweitzer3, K. M., Ossenkoppele3, G. J., Muller2, L. J., Heimans '&, J. J., & Huijgens3, P. C. (1994). Effects of the ACTH(4-9) analogue, ORG 2766, on vincristine cytotoxicity in two human lymphoma cell lines, U937 and U715. *British Journal of Cancer*, *69*, 497–501.

Kim, J., & Freeman, M. R. (2003). JNK/SAPK mediates doxorubicin-induced differentiation and apoptosis in MCF-7 breast cancer cells. *Breast Cancer Research and Treatment, 79,* 321-328.

Kim, S.-C., Hahn, J.-S., Min, Y.-H., Yoo, N.-C., Ko, Y.-W., & Lee, W.-J. (1999). Constitutive Activation of Extracellular Signal-Regulated Kinase in Human Acute Leukemias: Combined Role of Activation of MEK, Hyperexpression of Extracellular Signal-Regulated Kinase, and Downregulation of a Phosphatase, PAC1. *Blood*, *93*, 3893–3899.

Kirkwood, J. M., Bastholt, L., Robert, C., Sosman, J., Larkin, J., Hersey, P., … Dummer, R. (2012). Phase II, open-label, randomized trial of the MEK1/2 inhibitor selumetinib as monotherapy versus temozolomide in patients with advanced melanoma. *Clinical Cancer Research*, *18*, 555–567.

Kitagawa, D., Tanemura, S., Ohata, S., Shimizu, N., Seo, J., Nishitai, G., … Katada, T. (2002). Activation of extracellular signal-regulated kinase by ultraviolet is mediated through Src-dependent epidermal growth factor receptor phosphorylation. Its implication in an anti-apoptotic function. *Journal of Biological Chemistry, 277,* 366-371.

Knapper, S., Burnett, A. K., Littlewood, T., Kell, W. J., Agrawal, S., Chopra, R., … Small, D. (2006). A phase 2 trial of the FLT3 inhibitor lestaurtinib (CEP701) as first-line treatment for older patients with acute myeloid leukemia not considered fit for intensive chemotherapy. *Blood, 108,* 3262-3270.

Koeberle, S. C., Romir, J., Fischer, S., Koeberle, A., Schattel, V., Albrecht, W., … Laufer, S. A. (2012). Skepinone-L is a selective p38 mitogen-activated protein kinase inhibitor. *Nature Chemical Biology*, *8*, 141–143.

Kolch, W., Halasz, M., Granovskaya, M., & Kholodenko, B. N. (2015). The dynamic control of signal transduction networks in cancer cells. *Nature Reviews Cancer*, *15*, 515–527.

Kong, Q., Hua, H., Cui, A., Shao, T., Song, P., & Jiang, Y. (2014). SP600125 Induces Src and Type I IGF Receptor Phosphorylation Independent of JNK. *International Journal of Molecular Sciences*, *15*, 16246–16256.

Konopleva, M., Contractor, R., Tsao, T., Samudio, I., Ruvolo, P. P., Kitada, S., … Andreeff, M. (2006). Mechanisms of apoptosis sensitivity and resistance to the BH3 mimetic ABT-737 in acute myeloid leukemia. *Cancer Cell*, *10*, 343–345.

Kornblau, S. M., Womble, M., Qiu, Y. H., Jackson, C. E., Chen, W., Konopleva, M., … Andreeff, M. (2006). Simultaneous activation of multiple signal transduction pathways confers poor prognosis in acute myelogenous leukemia. *Blood*, *108*, 2358–2365.

Krilleke, D., Ucur, E., Pulte, D., Schulze-Osthoff, K., Debatin, K. M., & Herr, I. (2003). Inhibition of JNK signaling diminishes early but not late cellular stress-induced apoptosis. *International Journal of Cancer, 107,* 520-527.

Kuhl, N. M., & Rensing, L. (2000). Heat shock effects on cell cycle progression. *Cellular and Molecular Life Sciences*, *57*, 450–463.

Kuma, Y., Sabio, G., Bain, J., Shpiro, N., Márquez, R., & Cuenda, A. (2005). BIRB796 inhibits all p38 MAPK isoforms in vitro and in vivo. *Journal of Biological Chemistry*, *280*, 19472–19479.

Kumar, S., Jiang, M. S., Adams, J. L., & Lee, J. C. (1999). Pyridinylimidazole Compound SB203580 Inhibits the Activity but Not the Activation of p38 Mitogen-Activated Protein Kinase. *Biochemical and Biophysical Research Communities*, *263*, 825–831.

Kumar, S., McDonnell, P. C., Gum, R. J., Hand, A. T., Lee, J. C., & Young, P. R. (1997). Novel Homologues ofCSBP/p38 MAP Kinase: Activation, Substrate Specificity and Sensitivity to Inhibition by Pyridinyl Imidazoles. *Biochemical and Biophysical Research Communications*, *235*, 533–538.

Kurosu, T., Takahashi, Y., Fukuda, T., Koyama, T., Miki, T., & Mirua, O. (2005). p38 MAP kinase plays a role in G2 checkpoint activation and inhibits apoptosis of human B cell lymphoma cells treated with etoposide. *Apoptosis*, *10*, 1111–1120.

Kurzrock, R., Jordan, M. D., Gutterman, U., & Talpaz, M. D. (1988). The Molecular Genetics of Philadelphia Chromosome-Positive Leukemias. *The New Engalnd Journal of Medicine*, *319*, 990–998.

Kyriakis, J. M., & Avruch, J. (2001). Mammalian Mitogen-Activated Protein Kinase Signal Transduction Pathways Activated by Stress and

Inflammation. *Physiological Reviews*, *81*, 808–822.

Lagadinou, E. D., Ziros, P. G., Tsopra, O. A., Dimas, K., Kokkinou, D., Thanopoulou, E., … Zoumbos, N. C. (2008). c-Jun N-terminal kinase activation failure is a new mechanism of anthracycline resistance in acute myeloid leukemia. *Leukemia*, *22*, 1899–1908.

Lalaoui, N., Hänggi, K., Brumatti, G., Chau, D., Nguyen, N.-Y. N., Vasilikos, L., … Silke, J. (2016). Targeting p38 or MK2 Enhances the Anti-Leukemic Activity of Smac-Mimetics. *Cancer Cell*, *29*, 145–158.

Lange-Carter, C. A., Pleiman, C. M., Gardener, A. M., Blumer, K. J., & Johnson, G. L. (1993). A divergence in the MAP kinase regulatory network defined by MEK kinase and Raf. *Science*, *260*, 315–319.

Larrosa-Garcia, M., & Baer, M. R. (2017). FLT3 Inhibitors in Acute Myeloid Leukemia: Current Status and Future Directions. *Molecular Cancer Therapeutics*, *16*, 991–1001.

Lasunskaia, E. B., Fridlianskaia, I., Arnholdt, A. V., Kanashiro, M., Guzhova, I., & Margulis, B. (2010). Sub-lethal heat shock induces plasma membrane translocation of 70-kDa heat shock protein in viable, but not in apoptotic, U-937 leukaemia cells. *APMIS, 118,* 179-187.

Lavoie, H., & Therrien, M. (2015). Regulation of RAF protein kinases in ERK signalling. *Nature Reviews, 16,* 281-299.

Lawler, S., Fleming, Y., Goedert, M., & Cohen, P. (1998). Synergistic activation of SAPK1/JNK1 by two MAP kinase kinases in vitro. *Current Biology*, *8*, 1387–1390.

Le Dieu, R. L., Taussig, D. C., Ramsay, A. G., Mitter, R., Miraki-Moud, F., Fatah, R., … Gribben, J. G. (2009). Peripheral blood T cells in acute myeloid leukemia (AML) patients at diagnosis have abnormal phenotype and genotype and form defective immune synapses with AML blasts. *Blood*, *114*, 3909–3916.

Lee, J. W., Soung, Y. H., Park, W. S., Kim, S. Y., Nam, S. W., Min, W. S., … Lee, S. H. (2004). BRAF mutations in acute leukemias. *Leukaemia* , *18*, 170–172.

Leijen, S., Soetekouw, P. M. M. B., Jeffry E. T. R., Nicolson, M., Schellens, J. H. M., Learoyd, M., … Middleton, M. (2011). A phase I, open-label, randomized crossover study to assess the effect of dosing of the MEK 1/2 inhibitor Selumetinib (AZD6244; ARRY-142866) in the presence and absence of food in patients with advanced solid tumors. *Cancer Chemotherapy and Pharmacology*, *68*, 1619–1628.

Leonowens, C., Pendry, C., Bauman, J., Young, G. C., Ho, M., Henriquez, F., … Ouellet, D. (2014). Concomitant oral and intravenous pharmacokinetics of trametinib, a MEK inhibitor, in subjects with solid tumours. *British Journal of Clinical Pharmacology*, *78*, 524–532.

Leppä, S., Saffrich, R., Ansorge, W., & Bohmann, D. (1998). Differential

regulation of c-Jun by ERK and JNK during PC12 cell differentiation. *The EMBO Journal*, *17*, 4404–4413.

Leung, K. T., Li, K. K. H., Sun, S. S. M., Chan, P. K. S., Ooi, V. E. C., & Chiu, L. C. M. (2008). Activation of the JNK pathway promotes phosphorylation and degradation of BimEL - A novel mechanism of chemoresistance in T-cell acute lymphoblastic leukemia. *Carcinogenesis*, *29*, 544–551.

Levis, M. (2013). FLT3 mutations in acute myeloid leukemia: what is the best approach in 2013? *Hematology American Society of Hematology.. Education Program, 201,* 220-226.

Levis, M. (2013). Quizartinib in Acute Myeloid Leukemia. *Clinical Advances in Hematology and Oncology*, *11*, 586–588.

Levis, M., & Small, D. (2003). FLT3: ITDoes matter in leukemia. *Leukemia*, *17*, 1738–1752.

Li, C. Y., Lee, J. S., Ko, Y. G., Kim, J. I., & Seo, J. S. (2000). Heat shock protein 70 inhibits apoptosis downstream of cytochrome c release and upstream of caspase-3 activation. *Journal of Biological Chemistry, 275,* 25665-25671.

Li, F., Meng, L., Xing, H., Zhou, J., Huang, L., Xu, G., … Ma, D. (2006). Essential role of c-Jun-NH2-terminal kinase on synergy induction of apoptosis by TRAIL plus ADM in ADM resistant MCF-7/ADM cells. *Apoptosis*, *11*, 1239–1246.

Li, F., Meng, L., Zhou, J., Xing, H., Wang, S., Xu, G., … Ma, D. (2005). Reversing chemoresistance in cisplatin-resistant human ovarian cancer cells: A role of c-Jun NH2-terminal kinase 1. *Biochemical and Biophysical Research Communications*, *335*, 1070–1077.

Li, H., Liu, L., Xing, D., & Chen, W. R. (2010). Inhibition of the JNK/Bim pathway by Hsp70 prevents Bax activation in UV-induced apoptosis. *FEBS Letters*.

Li, J., & Yuan, J. (2008). Caspases in apoptosis and beyond. *Oncogene*, *27*, 6194–6206.

Li, Q., Song, X. M., Ji, Y. Y., & Xu, L. G. (2013). The dual mTORC1 and mTORC2 inhibitor AZD8055 inhibits head and neck squamous cell carcinoma cell growth in vivo and in vitro. *Biochemical and Biophysical Research Communications*, *440*, 701–706.

Li, Y., & Tergaonkar, V. (2016). Telomerase reactivation in cancers: Mechanisms that govern transcriptional activation of the wild-type vs. mutant TERT promoters. *Transcription*, *7*, 44–49.

Li, Z., Jiang, K., Zhu, X., Lin, G., Song, F., Zhao, Y., … Meng, S. (2016). Encorafenib (LGX818), a potent BRAF inhibitor, induces senescence accompanied by autophagy in BRAFV600E melanoma cells. *Cancer Letters*, *370*, 322–344.

Li, Z., Jiang, Y., Ulevitch, R. J., & Han, J. (1996). The Primary Structure of p38γ: A New Member of p38 Group of MAP Kinases. *Biochemical and Biophysical Research Communications*, *228*, 334–340.

Liang, D. C., Shih, L. Y., Fu, J. F., Li, H. Y., Wang, H. I., Hung, I. J., … Liu, H. C. (2006). K-Ras mutations and N-Ras mutations in childhood acute leukemias with or without mixed-lineage leukemia gene rearrangements. *Cancer, 106,* 950-956.

Lien, E. C., Lyssiotis, C. A., & Cantley, L. C. (2016). Metabolic Reprogramming by the PI3K-Akt-mTOR Pathway in Cancer. *Recent Results Cancer* , *207*, 39–72.

Liou, J.-T., Lin, C.-S., Liao, Y.-C., Ho, L.-J., Yang, S.-P., & Lai, J.-H. (2017). JNK/AP-1 activation contributes to tetrandrine resistance in T-cell acute lymphoblastic leukaemia. *Acta Pharmacologica*, *38*, 1171–1183.

Liu, J., & Lin, A. (2005). Role of JNK activation in apoptosis: A double-edged sword. *Cell Research*, *15*, 36–42.

Liu, R. Y., Fan, C., Liu, G., Olashaw, N. E., & Zuckerman, K. S. (2000). Activation of p38 Mitogen-activated Protein Kinase Is Required for Tumor Necrosis Factor-α-supported Proliferation of Leukemia and Lymphoma Cell Lines. *The Journal of Biological Chemistry*, *275*, 21086–21093.

Liu, W. H., & Chang, L. Sen. (2010). Suppression of ADAM17-mediated Lyn/Akt pathways induces apoptosis of human leukemia U937 cells: Bungarus multicinctus protease inhibitor-like protein-1 uncovers the cytotoxic mechanism. *Journal of Biological Chemistry, 285,* 30506-30515.

Liu, Y., & Cao, X. (2015). The origin and function of tumor-associated macrophages. *Cellular & Molecular Immunology*, *12*, 1–4.

Liu, Y. Q., You, S., Tashiro, S., Onodera, S., & Ikejima, T. (2005). Oridonin enhances phagocytosis of UV-irradiated apoptotic U937 cells. *Biological & Pharmaceutical Bulletin*, *28*, 461–467.

Lobo, V. J. S., Luquero, C. A., Alvarez-Vallina, L., Tipping, A. J., Viniegra, J. G., Losa, J. H., … Sanchez-Prieto, R. (2005). Modulation of the p38 MAPK (mitogen-activated protein kinase) pathway through Bcr/Abl: implications in the cellular response to Ara-C. *Biochemical Journal*, *387*, 231–238.

Lomax, M. E., Folkes, L. K., & O'Neill, P. (2013). Biological Consequences of Radiation-induced DNA Damage: Relevance to Radiotherapy. *Clinical Oncology*, *25*, 578–585.

LoRusso, P. M., Adjei, A. A., Varterasian, M., Gadgeel, S., Reid, J., Mitchell, D. Y., … Meyer, M. B. (2005). Phase I and pharmacodynamic study of the oral MEK inhibitor CI-1040 in patients with advanced malignancies. *Journal of Clinical Oncology*, *23*, 5281–5293.

LoRusso, P. M., Krishnamurthi, S. S., Rinehart, J. J., Nabell, L. M., Malburg, L., Chapman, P. B., … Ricart, A. D. (2010). Phase I pharmacokinetic and pharmacodynamic study of the oral MAPK/ERK kinase inhibitor PD-

0325901 in patients with advanced cancers. *Clinical Cancer Research*, *16*, 1924–1937.

Löwenberg, B., Licht, J. D., Tallman, M. S., Cornelissen, J. J., & Sternberg, D. W. (2005). Acute Myeloid Leukemia The Molecular Pathology of Acute Myeloid Leukemia. *Hematology*, *205*, 137–142.

Löwenberg, B., Pabst, T., Vellenga, E., Van Putten, W., Schouten, H. C., Graux, C., … Ossenkoppele, G. J. (2011). Cytarabine Dose for Acute Myeloid Leukemia, *11*, 1027-1036.

Luchetti, F., Betti, M., Canonico, B., Arcangeletti, M., Ferri, P., Galli, F., & Papa, S. (2009). ERK MAPK activation mediates the antiapoptotic signaling of melatonin in UVB-stressed U937 cells. *Free Radical Biology and Medicine*, *46*, 339–351.

Luchetti, F., Canonico, B., Curci, R., Battistelli, M., Mannello, F., Papa, S., … Falcieri, E. (2006). Melatonin prevents apoptosis induced by UV-B treatment in U937 cell line. *Journal of Pineal Research, 40,* 158-167.

Lunghi, P., Tabilio, A., Dall 'aglio, P., Ridolo, E., Carlo-Stella, C., Pelicci, P., & Bonati, A. (2003). Downmodulation of ERK activity inhibits the proliferation and induces the apoptosis of primary acute myelogenous leukemia blasts. *Leukemia*, *17*, 1783–1793.

Luskin, M. R., Lee, J.-W., Fernandez, H. F., Abdel-Wahab, O., Bennett, J. M., Ketterling, R. P., … Luger, S. M. (2016). Benefit of high-dose daunorubicin in AML induction extends across cytogenetic and molecular groups. *Blood*, *127*, 1551–1558.

Ma, F. Y., Flanc, R. S., Tesch, G. H., Han, Y., Atkins, R. C., Bennett, B. L., … Nikolic-Paterson, D. J. (2007). A Pathogenic Role for c-Jun Amino-Terminal Kinase Signaling in Renal Fibrosis and Tubular Cell Apoptosis. *Journal of the American Society of Nephrology*, *18*, 472–484.

Maha, A., Cheong, S.-K., Leong, C.-F., & Seiw, H.-F. (2009). Molecular responses during chemotherapy in acute myeloid leukemias in predicting poor - response to standard chemotherapy. *The Malaysian Journal of Pathology*, *31*, 81–91.

Manabe, A., Coustan-Smith, E., Behm, F. G., Raimondi, S. C., & Campana, D. (1992). Bone marrow-derived stromal cells prevent apoptotic cell death in B-lineage acute lymphoblastic leukemia. *Blood*, *79*, 2370–2377.

Manke, I. A., Nguyen, A., Lim, D., Stewart, M. Q., Elia, A. E. H., & Yaffe, M. B. (2005). MAPKAP Kinase-2 Is a Cell Cycle Checkpoint Kinase that Regulates the G2/M Transition and S Phase Progression in Response to UV Irradiation. *Molecular Cell*, *17*, 37–48.

Marfe, G., Pucci, B., De Martino, L., Fiorito, F., Di Stefano, C., Indelicato, M., … Tafani, M. (2009). Heat-shock pretreatment inhibits sorbitol-induced apoptosis in K562, U937 and HeLa cells. *International Journal of Cancer, 125,* 2077-2085.

Margolis, B., Skolnik, E. V, Margolis, B., & Skolnik, E. Y. (1994). Activation of Ras by Receptor Tyrosine Kinases. *Journall of the American Society of Nephrology*, *5*, 1288–1299.

Maroney, A. C., Finn, J. P., Connors, T. J., Durkin, J. T., Angeles, T., Gessner, G., … Vaugh, J. L. (2001). CEP-1347 (KT7515), a Semisynthetic Inhibitor of the Mixed Lineage Kinase Family. *The Journal of Biological Chemistry*, *276*, 25302–25308.

Maroney, A. C., Glicksman, M. A., Basma, A. N., Walton, K. M., Knight, E. J., Murphy, C. A., … Dionne, C. A. (1998). Motorneuron apoptosis is blocked by CEP-1347 (KT 7515), a novel inhibitor of the JNK signalling pathway. *The Journal of Neuroscience*, *18*, 104–111.

Masson, K., & Rönnstrand, L. (2009). Oncogenic signaling from the hematopoietic growth factor receptors c-Kit and Flt3. *Cellular Signalling, 21,* 1717-1726.

Matsuda, S., Kawasaki, H., Moriguchi, T., Gotoh, Y., & Nishida, E. (1995). Activation of protein kinase cascades by osmotic shock. *Journal of Biological Chemistry, 270,* 12781-12786.

Mauro, A., Ciccarelli, C., De Cesaris, P., Scoglio, A., Bouché, M., Molinaro, M., … Zani, B. M. (2002). PKCalpha-mediated ERK, JNK and p38 activation regulates the myogenic program in human rhabdomyosarcoma cells. *Journal of Cell Science*, *115*, 3587–3599.

Mayer, I. A., Verma, A., Grumbach, I. M., Uddin, S., Lekmine, F., Ravandi, F., … Platanias, L. C. (2001). The p38 MAPK Pathway Mediates the Growth Inhibitory Effects of Interferon-α in BCR-ABL-expressing Cells. *The Journal of Biological Chemistry*, *276*, 2570–2577.

Mc Gee, M. K., Campiani, G., Nacci, V., Lawler, M., Williams, D. C., & Zisterer, D. M. (2002). Activation of the c-Jun N-terminal Kinase (JNK) Signaling Pathway Is Essential during PBOX-6-induced Apoptosis in Chronic Myelogenous Leukemia (CML) Cells. *The Journal of Biological Chemistry*, *277*, 18383–18389.

Messoussi, A., Feneyrolles, C., Bros, A., Deroide, A., Dayde-Cazals, B., Cheve, G., … Yasri, A. (2014). Recent Progress in the Design, Study, and Development of c-Jun N-Terminal Kinase Inhibitors as Anticancer Agents. *Chemistry & Biology*, *21*, 1433–1443.

Milella, M., Kornblau, S. M., Estrov, Z., Carter, B. Z., Lapillonne, H., Harris, D., … Andreeff, M. (2001). Therapeutic targeting of the MEK/MAPK signal transduction module in acute myeloid leukemia. *Journal of Clinical Investigation, 108,* 851-859.

Millwards, T. A., Zolnierowicz, S., & Hemmings, B. A. (1999). Regulation of protein kinase cascades by protein phosphatase 2A. *Trends in Biochemical Sciences*, *24*, 186–191.

Mingo-Sion, A. M., Marietta, P. M., Koller, E., Wolf, D. M., Van, C. L., & Berg,

D. (2004). Inhibition of JNK reduces G2/M transit independent of p53, leading to endoreduplication, decreased proliferation, and apoptosis in breast cancer cells. *Oncogene*, *23*, 596–604.

Momparler, R. L. (2013). Optimization of cytarabine (ARA-C) therapy for acute myeloid leukemia. *Experimental Haematology & Oncology, 2*, 1-5.

Moon, D. O., Choi, Y. H., & Kim, G. Y. (2011). Role of p21 in SP600125-induced cell cycle arrest, endoreduplication, and apoptosis. *Cellular and Molecular Life Sciences, 68,* 3249-3260.

Moon, D. O., Kim, M. O., Choi, Y. H., Kim, N. D., Chang, J. H., & Kim, G. Y. (2008). Bcl-2 overexpression attenuates SP600125-induced apoptosis in human leukemia U937 cells. *Cancer Letters*, *264*, 316–325.

Moon, D. O., Kim, M. O., Kang, C. H., Lee, J. D., Choi, Y. H., & Kim, G. Y. (2009). JNK inhibitor SP600125 promotes the formation of polymerized tubulin, leading to G2/M phase arrest, endoreduplication, and delayed apoptosis. *Experimental & Molecular Medicine*, *41*, 665–77.

Morgan, M. A., Dolp, O., & Reuter, C. W. M. (2001). Cell-cycle–dependent activation of mitogen-activated protein kinase kinase (MEK-1/2) in myeloid leukemia cell lines and induction of growth inhibition and apoptosis by inhibitors of RAS signaling. *Blood*, *97*, 1823–1834.

Moriguchi, T., Kuroyanagi, N., Yamaguchiţ, K., Gotoh, Y., Irieţ, K., Kano, T., … Hagiwara, M. (1996). A Novel Kinase Cascade Mediated by Mitogen-activated Protein Kinase Kinase 6 and MKK3. *The Journal of Biological Chemistry*, *271*, 13675–13679.

Morris, E. J., Jha, S., Restaino, C. R., Dayananth, P., Zhu, H., Cooper, A., … Samatar, A. A. (2013). Discovery of a novel ERK inhibitor with activity in models of acquired resistance to BRAF and MEK inhibitors. *Cancer Discovery*, *3*, 742–750.

Morrison, D. K. (2012). MAP kinase pathways. *Cold Spring Harbor Perspectives in Biology*.

Morsi, M. G., Gharabawy, M. M., Hamed, N. A., El Sawy, M, M., Seada, N. M. A., & Hashad, R. A. (2016). Quantitative Expression of Toll-Like Receptors TLR-7 and TLR-9 on Peripheral Blood Mononuclear Cells in Leukemias. *Journal of Haematology*, *5*, 17–24.

Mosser, D. D., Caron, A. W., Bourget, L., Denis-Larose, C., & Massie, B. (1997). Role of the Human Heat Shock Protein hsp70 in Protection against Stress-Induced Apoptosis. *Molecular and Cellular Biology*, *17*, 5317–5327.

Mosser, D. D., Caron, A. W., Bourget, L., Meriin, A. B., Sherman, M. Y., Morimoto, R. I., & Massie, B. (2000). The Chaperone Function of hsp70 is Required for Protection against Stress-Induced Apoptosis. *Molecular and Cellular Biology*, *20*, 7146–7159.

Moudi, M., Go, R., Yien, C., & Nazre, M. (2013). Vinca Alkaloids. *International*

Journal of Preventive Medicine, *4*, 1231–1235.

Muniyappa, H., & Das, K. C. (2008). Activation of c-Jun N-terminal kinase (JNK) by widely used specific p38 MAPK inhibitors SB202190 and SB203580: A MLK-3-MKK7-dependent mechanism. *Cellular Signalling, 20,* 675-683.

Munshi, A., & Ramesh, R. (2013). MItogen-activated protein kinases and Their Role in Radiation Response. *Genes & Cancer*, *4*, 401–408.

Muscarella, D. E., & Bloom, S. E. (2008). The contribution of c-Jun N-terminal kinase activation and subsequent Bcl-2 phosphorylation to apoptosis induction in human B-cells is dependent on the mode of action of specific stresses. *Toxicology and Applied Pharmacology*.

Negrini, S., Gorgoulis, V. G., & Halazonetis, T. D. (2010). Genomic instability — an evolving hallmark of cancer. *Nature Reviews Molecular Cell Biology*, *11*, 220–228.

Nemoto, S., Xiang, J., Huang, S., & Lin, A. (1998a). Induction of Apoptosis by SB202190 through Inhibition of p38β Mitogen-activated Protein Kinase*. *The Journal of Biological Chemistry*, *273*, 16415–16420.

Neri, A., Knowlest, D. M., Greco, A., Mccormickt, F., & Dalla-Favera, R. (1988). Analysis of RAS oncogene mutations in human lymphoid malignancies. *Medical Sciences*, *85*, 9268–9272.

Neviani, P., Santhanam, R., Trotta, R., Notari, M., Blaser, B. W., Liu, S., … Perrotti, D. (2005). The tumor suppressor PP2A is functionally inactivated in blast crisis CML through the inhibitory activity of the BCR/ABL-regulated SET protein. *Cancer Cell, 8, 355-368*.

New, L., Jiang, Y., Zhao, M., Liu, K., Zhu, W., Flood, L. J., … Han, J. (1998). PRAK, a novel protein kinase regulated by the p38 MAP kinase. *The EMBO Journal*, *17*, 3372–3384.

Ng, D. C. H., & Bogoyevitch, M. A. (2000). The mechanism of heat shock activation of ERK mitogen-activated protein kinases in the interleukin 3-dependent proB cell line BaF3. *Journal of Biological Chemistry,275,* 40856-40866.

Ngoei, K. R. W., Catimel, B., Church, N., Lio, D. S., Dogovski, C., & Perugini. (2011). Characterization of a novel JNK (c-Jun N-terminal kinase) inhibitory peptide. *Biochemical Journal*, *434*, 399–413.

Nica, A. F., Tsao, C. C., Watt, J. C., Jiffar, T., Kurinna, S., Jurasz, P., … Ruvolo, P. (2008). Ceramide Promotes Apoptosis in Chronic Myelogenous Leukemia-Derived K562 Cells by a Mechanism Involving Caspase-8 and JNK. *Cell Cycle*, *7*, 3362–3370.

Nilles, N., & Fahrenkrog, B. (2017). Taking a Bad Turn: Compromised DNA Damage Response in Leukemia. *Cells*, *6*, 1–18.

Nishida, N., Yano, H., Nishida, T., Kamura, T., & Kojiro, M. (2006).

Angiogenesis in cancer. *Vascular Health and Risk Management*, *2*, 213–219.

Nishii, K., Katayama, N., Miwa, H., Shikami, M., Masuya, M., Shiku, H., & Kita, K. (1999). Survival of human leukaemic B-cell precursors is supported by stromal cells and cytokines: Association with the expression of bcl-2 protein. *British Journal of Haematology*, *105*, 701–710.

Ohori, M., Kinoshita, T., Okubo, M., Sato, K., Yamazaki, A., Arakawa, H., … Fujii, T. (2005). Identification of a selective ERK inhibitor and structural determination of the inhibitor-ERK2 complex. *Biochemical and Biophysical Research Communications*, *336*, 357–363.

Okada, M., Kuramoto, K., Takeda, H., Watarai, H., Sakaki, H., Seino, S., … Kitanaka, C. (2016). The novel JNK inhibitor AS602801 inhibits cancer stem cells in vitro and in vivo. *Oncotarget*, *7*, 27021–27032.

Omerovic, J., Laude, A. J., & Prior, I. A. (2007). Ras proteins: Paradigms for compartmentalised and isoform-specific signalling. *Cellular and Molecular Life Sciences, 64,* 2575-2589.

Onida, F., Ball, G., Kantarjian, H. M., Smith, T. L., Glassman, A., Albitar, M., … Beran, M. (2002). Characteristics and outcome of patients with Philadelphia chromosome negative, bcr/abl negative chronic myelogenous leukemia. *Cancer, 95,* 1673-1684.

Ono, K., & Han, J. (2000). The p38 signal transduction pathway Activation and function. *Cellular Signalling*, *12*, 1–13.

Ortega-Pérez, I., Cano, E., Were, F., Villar, M., Vázquez, J., & Redondo, J. M. (2005). c-Jun N-terminal kinase (JNK) positively regulates NFATc2 transactivation through phosphorylation within the N-terminal regulatory domain. *Journal of Biological Chemistry, 280,* 20867-20878.

Ozgen, U., Savaan, S., Stout, M., Buck, S., & Ravindranath, Y. (2000). Further elucidation of mechanism of resistance to vincristine in myeloid cells: role of hypochlorous acid in degradation of vincristine by myeloperoxidase. *Leukemia*, *14*, 47–51.

Panaretakis, T., Laane, E., Pokrovskaja, K., Bjö, A.-C., Moustakas, A., Zhivotovsky, B., … Grandér, D. (2005). Doxorubicin Requires the Sequential Activation of Caspase-2, Protein Kinase C□, and c-Jun NH 2 -terminal Kinase to Induce Apoptosis □ D. *Molecular Biology of the Cell*, *16*, 3821–3831.

Panayiotidis, P., Jones, D., Ganeshaguru, K., Foroni, L., & Hoffbrand, A. V. (1996). Human bone marrow stromal cells prevent apoptosis and support the survival of chronic lymphocytic leukaemia cells in vitro. *British Journal of Haematology*, *92*, 97–103.

Pargellis, C., Tong, L., Chuchill, L., Cirillo, P. F., Gilmore, T., Graham, A. G., … Regan, J. (2002). Inhibition of p38 MAP kinase by utilizing a novel allosteric binding site. *Nature Structural and Molecular Biology*, *9*, 268–

272.

Park, C., Jin, C. Y., Kim, G. Y., Choi, W., Kwon, T. K., Choi, B. T., … Choi, Y. H. (2008). Induction of apoptosis by esculetin in human leukemia U937 cells through activation of JNK and ERK. *Toxicology and Applied Pharmacology*, *227*, 219–228.

Park, H. S., Hwang, H. J., Kim, G.-Y., Cha, H.-J., Kim, W.-J., Kim, N. D., … Choi, Y. H. (2013). Induction of Apoptosis by Fucoidan in Human Leukemia U937 Cells through Activation of p38 MAPK and Modulation of Bcl-2 Family. *Marine Drugs*, *11*, 2347–2364.

Park, H. S., Lee, J. S., Huh, S. H., Seo, J. S., & Choi, E. J. (2001). Hsp27 functions as a natural inhibitory protein of c-Jun N-terminal kinase. *EMBO Journal*, *20*, 446–456.

Parmar, S., Katsoulidis, E., Verma, A., Li, Y., Sassano, A., Lal, L., … Platanias, L. C. (2004). Role of the p38 mitogen-activated protein kinase pathway in the generation of the effects of imatinib mesylate (STI571) in BCR-ABL-expressing cells. *Journal of Biological Chemistry*, *279*, 25345–25352.

Paul, C., Manero, F., Gonin, S., Kretz-Remy, C., Virot, S., & Arrigo, A. P. (2002). Hsp27 as a negative regulator of cytochrome c release. *Molecular and Cellular Biology*, *22*, 816–834.

Payne, D. M., Rossomando, A. J., Martino, P., Erickson, A. K., Her, J., Shabanowitz, J., … Sturgill, T. W. (1991). Identification of the regulatory phosphorylation sites in pp42/mitogen-activated protein kinase (MAP kinase). *The EMBO Journal*, *10*, 885–892.

Pedersen, I. M., Buhl, A. M., Klausen, P., Geisler, C. H., & Jurlander, J. (2002). The chimeric anti-CD20 antibody rituximab induces apoptosis in B-cell chronic lymphocytic leukemia cells through a p38 mitogen activated protein–kinase–dependent mechanism. *Blood*, *99*, 1314–1319.

Peng, Z., Liu, D., Yao, Y., Feng, X., Huang, X., Tang, Y., … Wang, X. (2016). Paclitaxel induces apoptosis in leukemia cells through a JNK activation-dependent pathway. *Genetics and Molecular Research*, *15*, 1-13.

Perim, A., Amarante, M. K., Guembarovski, R. L., Eduadro, C., Oliveria, C., & Watanabe, M. A. E. (2015). CXCL12/CXCR4 axis in the pathogenesis of acute lymphoblastic leukemia (ALL): a possible therapeutic target. *Cellular and Molecular Life Sciences*, *72*, 1715–1723.

Perona, R. (2006). Cell signalling: Growth factors and tyrosine kinase receptors. *Clinical and Translational Oncology, 8,* 77-82*.*

Phan, L. M., Yeung, S.-C. J., & Lee, M.-H. (2014). Cancer metabolic reprogramming: importance, main features and potentials for precise targeted anti-cancer therapies. *Cancer Biology & Medicine*, *11*, 1–19.

Pietersma, A., Tilly, B. C., Gaestel, M., Jong, N., Lee, J. C., Koster, J. F., & Sluiter, W. (1997). P38 Mitogen Activated Protein Kinase Regulates Endothelial VCAM-1 Expression at the Post-transcriptional Level.

Biochemical and Biophysical Research Communications, *230*, 44–48.

Pikor, L., Thu, K., Vucic, E., & Lam, W. (2013). The detection and implication of genome instability in cancer. *Cancer and Metastasis Reviews*, *32*, 341–352.

Platanias, L. C. (2003). Map kinase signaling pathways and hematologic malignancies. *Blood*, *101*, 4667–4679.

Plotnikov, A., Zehorai, E., Procaccia, S., & Seger, R. (2011). The MAPK cascades: Signaling components, nuclear roles and mechanisms of nuclear translocation. *Biochimica et Biophysica Acta, 1813,* 1619-1633.

Porras, A., & Guerrero, C. (2011). Role of p38α in apoptosis: implication in cancer development and therapy. *Atlas of Genetics and Cytogenetics in Oncology and Haematology*, *15*, 316–326.

Poulikakos, P. I., Zhang, C., Bollag, G., Shokat, K. M., & Rosen, N. (2010). RAF inhibitors transactivate RAF dimers and ERK signalling in cells with wild-type BRAF. *Nature*, *464*, 427–431.

Prasad, S., Gupta, S. C., & Tyagi, A. K. (2017). Reactive oxygen species (ROS) and cancer: Role of antioxidative nutraceuticals. *Cancer Letters, 387,* 95-105.

Pratz, K. W., Cho, E., Levis, M. J., Karp, J. E., Gore, S. D., Mcdevitt, M., … Smith, B. D. (2010). A pharmacodynamic study of sorafenib in patients with relapsed and refractory acute leukemias. *Leukemia*, *24*, 1437–1444.

Prior, I. A., Lewis, P. D., & Mattos, C. (2012). A comprehensive survey of ras mutations in cancer. *Cancer Research*, *72*, 2457–2467.

Prokhorova, I. V, Akulich, K. A., Makeeva, D. S., Osterman, I. A., Skvortsov, D. A., Sergiev, P. V, … Dmitriev, S. E. (2016). Amicoumacin A induces cancer cell death by targeting the eukaryotic ribosome. *Nature, 6,* 1-10.

Proskuryakov, A. Y., Konoplyannikov, A. G., & Gabai, V. L. (2003). Necrosis: a specific form of programmed cell death? *Experimental Cell Research*, *283*, 1–16.

Rahmani, M., Anderson, A., Habibi, J. R., Crabtree, T. R., Mayo, M., Harada, H., … Grant, S. (2009). The BH3-only protein Bim plays a critical role in leukemia cell death triggered by concomitant inhibition of the PI3K/Akt and MEK/ERK1/2 pathways. *Blood*, *114*, 4507–4516.

Raitano, A. B., Halpern, J. R., Hambuch, T. M., & Sawyers, C. L. (1995). The Bcr-Abl leukemia oncogene activates Jun kinase and requires Jun for transformation (chronic myelogenous leukemia/mitogen-activated protein kinase). *Proceedings of the National Academy of Sciences*, *92*, 11746–11750.

Raman, M., Chen, W., & Cobb, M. (2007). Differential regulation and properties of MAPKs. *Oncogene*, *26*, 3100–3112.

Ramaswamy, K., Spitzer, B., Kentsis, A., Ruvolo, P., & Gutierrez, A. (2015). Therapeutic re-activation of protein phosphatase 2A in acute myeloid leukemia. *Frontiers in Oncology, 5*, 1-5.

Ramos, J. W. (2008). The regulation of extracellular signal-regulated kinase (ERK) in mammalian cells. *The International Journal of Biochemistry & Cell Biology*, *40*, 2707–2719.

Rane, M. J., Pan, Y., Singh, S., Powell, D. W., Wu, R., Cummins, T., … Klein, J. B. (2003). Heat shock protein 27 controls apoptosis by regulating Akt activation. *The Journal of Biological Chemistry*, *278*, 27828–27835.

Regan, J., Pargellis, C. A., Cirillo, P., Gilmore, T., Hickey, E. R., Peet, G. W., … Moss, N. (2003). The kinetics of binding to p38 MAP kinase by analogues of BIRB 796. *Bioorganic and Medicinal Chemistry Letters*, *13*, 3101–3104.

Reisz, J. A., Bansal, N., Qian, J., Zhao, W., & Furdui, C. M. (2014). Effects of Ionizing Radiation on Biological Molecules—Mechanisms of Damage and Emerging Methods of Detection. *Antioxidants & Redox Signaling*, *21*, 260–292.

Remy, G., Risco, A. M., Iñesta-Vaquera, F. A., González-Terán, B., Sabio, G., Davis, R. J., & Cuenda, A. (2010). Differential activation of p38MAPK isoforms by MKK6 and MKK3. *Cellular Signalling*, *22*, 660–667.

Reuter, C. W. M., Morgan, M. A., & Bergmann, L. (2000). Targeting the Ras signaling pathway: a rational, mechanism-based treatment for hematologic malignancies? *Blood*, *96*, 1655–1669.

Ricciardi, M., Mcqueen, T., Chism, D., Milella, M., Estey, E., Kaldjian, E., … Andreeff, M. (2005). Quantitative single cell determination of ERK phosphorylation and regulation in relapsed and refractory primary acute myeloid leukemia. *Leukemia*, *19*, 1543–1549.

Rice, K. D., Aay, N., Anand, N. K., Blazey, C. M., Bowles, O. J., Bussenius, J., … Johnston, S. (2012). Novel carboxamide-based allosteric MEK inhibitors: Discovery and optimization efforts toward XL518 (GDC-0973). *ACS Medicinal Chemistry Letters*, *3*, 416–421.

Rieber, M., & Rieber, M. S. (2006). Signalling responses linked to betulinic acid-induced apoptosis are antagonized by MEK inhibitor U0126 in adherent or 3D spheroid melanoma irrespective of p53 status. *International Journal of Cancer, 118,* 1135-1143.

Riedhammer, C., Halbirtter, D., & Weissert, R. (2014). *Peripheral Blood Mononuclear Cells: Isolation, Freezing, Thawing, and Culture*. Human Press, New York, NY.

Riedl, S. J., & Shi, Y. (2004). Molecular mechanisms of caspase regulation during apoptosis. *Nature Reviews Molecular Cell Biology*, *5*, 897–907.

Ringshausen, I., Dechow, T., Schneller, F., Weick, K., Oelsner, M., Peschel, C., & Decker, T. (2004). Constitutive activation of the MAPkinase p38 is

critical for MMP-9 production and survival of B-CLL cells on bone marrow stromal cells. *Leukemia*, *18*, 1964–1970.

Robarge, K., Schwarz, J., Blake, J., Burkard, M., Chan, J., Chen, H., … Moffat, J. G. (2014). Discovery of GDC-0994, a potent and selective ERK1/2 inhibitor in early clinical development. *Experimental and Molecular Therapeutics*, *74*, 1.

Rogatsky, I., Logan, S. K., Garabedian, M. J., & Yamamoto, K. R. (1998). Antagonism of glucocorticoid receptor transcriptional activation by the c-Jun N-terminal kinase. *Biochemistry*, *95*, 2050–2055.

Roskoski, R. (2012). MEK1/2 dual-specificity protein kinases: Structure and regulation. *Biochemical and Biophysical Research Communications, 417,* 5-10.

Rouse, J., Cohen, P., Trigon, S., Morange, M., Alonso-Llamazares, A., Zamanillo, D., … Nebreda, A. (1994). A novel kinase cascade triggered by stress and heat shock that stimulates MAPKAP kinase-2 and phosphorylation of the small heat shock proteins. *Cell*, *78*, 1027–1037.

Russell, M., Lange-Carter, C. A., & Johnson, G. L. (1995). Direct Interaction between Ras and the Kinase Domain or Mitogen-activated Protein Kinase Kinase Kinase (MEKK1). *The Journal of Biological Chemistry*, *270*, 11757–11760.

Salerni, B. L., Bates, D. J., Albershardt, T. C., Lowrey, C. H., & Eastman, A. (2010). Vinblastine Induces Acute, Cell Cycle Phase–Independent Apoptosis in Some Leukemias and Lymphomas and Can Induce Acute Apoptosis in Others when Mcl-1 Is Suppressed. *Molecular Cancer Therapeutics*, *9*, 791–802.

Salmeron, A., Ahmad, T. B., Carlile, G. W., Pappin ', D., Narsimhan R P, & Ley, S. C. (1996). Activation of MEK-1 and SEK-1 by Tpl-2 proto-oncoprotein, a novel MAP kinase kinase kinase. *The EMBO Journal*, *15*, 817–826.

Samali, A., & Cotter, T. G. (1996). Heat Shock Proteins Increase Resistance to Apoptosis. *Experimental Cell Research*, *223*, 163–170.

Samatar, A. A., & Poulikakos, P. I. (2014). Targeting RAS–ERK signalling in cancer: promises and challenges. *Nature*, *13,* 928-942.

Santarpia, L., Lippman, S. L., & El-Naggar, A. K. (2012). Targeting the Mitogen-Activated Protein Kinase RAS-RAF Signaling Pathway in Cancer Therapy. *Expert Opinion on Therapeutic Targets*, *16*, 103–119.

Santivasi, W. L., & Xia, F. (2014). Ionizing radiation-induced DNA damage, response, and repair. *Antioxidants & Redox Signaling*, *2*, 251–259.

Saporito, M. S., Hudkins, R. L., & Maroney, A. C. (2002). Discovery of CEP-1347/KT-7515, an inhibitor of the JNK/SAPK pathway for the treatment of neurodedenerative diseases. *Progress in Medicinal Chemistry*, *40*, 23–62.

Saraste, A., & Pulkki, K. (2000). Morphologic and biochemical hallmarks of apoptosis. *Cardiovascular Research*, *45*, 528–537.

Sato, S., Fujita, N., & Tsuruo, T. (2000). Modulation of Akt kinase activity by binding to Hsp90. *Proceedings of the Natural Academy of Science*, *97*, 10832–10837.

Saunders, P., Cisterne, A., Weiss, J., Bradstock, K. F., & Bendall, L. J. (2011). The mammalian target of rapamycin inhibitor RAD001 (everolimus) synergizes with chemotherapeutic agents, ionizing radiation and proteasome inhibitors in pre-B acute lymphocytic leukemia. *Haematologica, 96,* 69-77.

Schwartz, H. S., Preisler, H. D., & Kanter, P. M. (1981). DNA damage in AML cells exposed to adriamycin; correlations with clinical response to therapy. *Leukemia Research*, *5*, 263–366.

Sebolt-Leopold, J. S., Dudley, D., Herrera, R., Van Becelaere, K., Wiland, A., Gowan, R. C., … Saltiel, A. (1999). Blockade of the MAP kinase pathway supresses growth of colon tumors in vivo. *Nature Medicine*, *5*, 810–816.

Segawa, K., & Nagata, S. (2015). An Apoptotic “Eat Me” Signal: Phosphatidylserine Exposure. *Trends in Cell Biology*, *25*, 639–650.

Seki, E., Brenner, D. A., & Karin, M. (2012). A Liver Full of JNK: Signaling in Regulation of Cell Function and Disease Pathogenesis, and Clinical Approaches. *Gastroenterology*, *143*, 307–320.

Sever, R., & Brugge, J. S. (2015). Signal transduction in cancer. *Cold Spring Harbor Perspectives in Medicine*, *5*, 1–21.

Shaul, Y. D., & Seger, R. (2007). The MEK/ERK cascade: From signaling specificity to diverse functions. *Biochimica et Biophysica Acta, 1773,* 1213-1226.

Shaulian, E., & Karin, M. (2001). AP-1 in cell proliferation and survival. *Oncogene*, *20*, 2390–2400.

Shen, Z. (2011). Genomic instability and cancer: An introduction. *Journal of Molecular Cell Biology*, *3*, 1–3.

Sherr, C. J., & McCormick, F. (2002). The RB and p53 pathways in cancer. *Cancer Cell*, *2*, 103–112.

Shiah, S.-G., Chuang, S.-E., & Kuo, M.-L. (2001). Involvement of Asp-Glu-Val-Asp-Directed, Caspase-Mediated Mitogen-Activated Protein Kinase Kinase 1 Cleavage, c-Jun N-Terminal Kinase Activation, and Subsequent Bcl-2 Phosphorylation for Paclitaxel-Induced Apoptosis in HL-60 Cells. *Molecular Pharmacology*, *59*, 254–262.

Shimizu, S., Yoshida, T., Tsujioka, M., & Arakawa, S. (2014). Autophagic cell death and cancer. *International Journal of Molecular Sciences, 15,* 3145-3153.

Shin, S. W., Park, C. I., Yang, C. H., & Park, J. (2008). Protective Effect of Rehmannia glutinosa on the UV-Induced Apoptosis in U937 Cells. *Journal of Chinese Medicine*, *36*, 1159–1170.

Siddiqui, M. A., & Reddy, P. A. (2010). Small molecule JNK (c-Jun N-terminal kinase) inhibitors. *Journal of Medicinal Chemistry*, *53*, 3005–3012.

Silva, M. T. (2010). Secondary necrosis: The natural outcome of the complete apoptotic program. *FEBS Letters*, *584*, 4491–4499.

Smith, A. M., Dun, M. D., Lee, E. M., Harrison, C., Kahl, R., Flanagan, H., … Verrills, N. M. (2016). Activation of protein phosphatase 2A in FLT3+ acute myeloid leukemia cells enhances the cytotoxicity of FLT3 tyrosine kinase inhibitors. *Oncotarget*, *7*, 47465–47478.

Smith, B. D., Levis, M., Beran, M., Giles, F., Kantarjian, H., Berg, K., … Small, D. (2004). Single-agent CEP-701, a novel FLT3 inhibitor, shows biologic and clinical activity in patients with relapsed or refractory acute myeloid leukemia. *Blood*, *103*, 3669–3676.

Smith, J., Tho, L. M., Xu, N., & Gillespie, D. A. (2010). The ATM-Ch2 and ATR-Ch1 Pathay in DNA Damage Signaling and Cancer. *Advances in Cancer Research*, *108*, 73–112.

Smith, M. L., Snaddon, J., Neat, M., Cambal-Parrales, M., Arch, R., Lister, T. A., & Fitzgibbon, J. (2003). Mutation of BRAF is uncommon in AML FAB type M1 and M2. *Leukemia*, *17*, 274–275.

Spiegel, A., Kollet, O., Peled, A., Abel, L., Nagler, A., Bielorai, B., … Lapidot, T. (2004). Unique SDF-1-induced activation of human precursor-B ALL cells as a result of altered CXCR4 expression and signaling. *Blood*, *103*, 2900–2907.

Stadheim, T. A., & Kucera, G. L. (2002). c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) is required for mitoxantrone- and anisomycin-induced apoptosis in HL-60 cells. *Leukemia Research*, *26*, 55–65.

Stadheim, T. A., Xiao, H., & Eastman, A. (2001). Inhibition of extracellular signal-regulated kinase (ERK) mediates cell cycle phase independent apoptosis in vinblastine-treated ML-1 cells. *Cancer Research*, *61*, 1533–1540.

Stadtmauer, E. A. (2002). Trials with Gemtuzumab Ozogamicin (MylotargÂ®) Combined with Chemotherapy Regimens in Acute Myeloid Leukemia. *Clinical Lymphoma*, *2*, 24–28.

Staples, C. J., Owens, D. M., Maier, J. V., Cato, A. C. B., & Keyse, S. M. (2010). Cross-talk between the p38α and JNK MAPK pathways mediated by MAP kinase phosphatase-1 determines cellular sensitivity to UV radiation. *Journal of Biological Chemistry*, *285*, 25928–25940.

Stephen, W. G., & Green, D. R. (2010). Mitochondria and cell death: outer membrane permeabilization and beyon. *Nature Reviews Molecular Cell*

Biology, *11*, 621–632.

Stone, A. A., & Chambers, T. C. (2000). Microtubule inhibitors elicit differential effects on MAP kinase (JNK, ERK, and p38) signaling pathways in human KB-3 carcinoma cells. *Experimental Cell Research*, *254*, 110–119.

Stone, R. M., DeAngelo, D. J., Klimek, V., Galinsky, I., Estey, E., Nimer, S. D., … Griffin, J. D. (2005). Patients with acute myeloid leukemia and an activating mutation in FLT3 respond to a small-molecule FLT3 tyrosine kinase inhibitor, PKC412. *Blood, 105,* 54-60.

Storch, K., Gehringer, M., Baur, B., & Laufer, S. A. (2014). Metabolism of a novel skepinone-L-like p38 mitogen-activated protein kinase inhibitor. *Medicinal Chemical Communications*, *5*, 808–815.

Stuart, D. D., Li, N., Poon, D. J., Aardalen, K., Kaufman, S., Merritt, H., … Pryer, N. (2012). Preclinical profile of LGX818: A potent and selective RAF kinase inhibitor. *Experimental and Molecular Therapeutics*, *72*, 1.

Sturgill, T. W., Ray, L. B., Erikson, E., & Maller, J. L. (2002). Insulin-stimulated MAP-2 kinase phosphorylates and activates ribosomal protein S6 kinase II. *Nature*, *334*, 715–718.

Su, X., Wang, X., Zhang, K., Yang, S., Xue, Q., Wang, P., & Liu, Q. (2014). ERK inhibitor U0126 enhanced SDT-induced cytotoxicity of human leukemia U937 cells. *General Physiology and Biophysics, 33,* 295-309.

Tallman, M. S., Gilliland, D. G., & Rowe, J. M. (2005). Drug therapy for acute myeloid leukemia. *Blood, 106,* 1154-1163.

Tan, W., Yu, H.-G., & Luo, H.-S. (2014). Inhibition of the p38 MAPK pathway sensitizes human gastric cells to doxorubicin treatment in vitro and in vivo. *Molecular Medicine Reports*, *10*, 3275–3281.

Tauchi, T., Shin-Ya, K., Sashida, G., Sumi, M., Okabe, S., Ohyashiki, J., & Ohyashiki, K. (2006). Telomerase inhibition with a novel G-quadruplex-interactive agent, telomestatin: in vitro and in vivo studies in acute leukemia. *Oncogene*, *25*, 5719–5725.

Thornton, T. M., & Rincon, M. (2009). Non-classical p38 map kinase functions: Cell cycle checkpoints and survival. *International Journal of Biological Sciences*, *5*, 44–52.

Tiacci, E., Park, J. H., De Carolis, L., Chung, S. S., Broccoli, A., Scott, S., … Tallman, M. S. (2015). Targeting Mutant BRAF in Relapsed or Refractory Hairy-Cell Leukemia. *The New England Journal of Medicine*, *373*, 1733–1747.

Tiacci, E., Trifonov, V., Schiavoni, G., Holmes, A., Kern, W., Paola Martelli, M., … Falini, B. (2011). BRAF Mutations in Hairy-Cell Leukemia. *N Engl J Med*, *364*, 2305–15.

Törocsik, B., & Szeberényi, J. (2000). Anisomycin uses multiple mechanisms to stimulate mitogen-activated protein kinases and gene expression and

to inhibit neuronal differentiation in PC12 phaeochromocytoma cells. *European Journal of Neuroscience*, *12*, 527–532.

Tournier, C. (2013). The 2 Faces of JNK Signaling in Cancer. *Genes & Cancer*, *4*, 397–400.

Towatri, M., Lida, H., Tanimoti, M., Iwata, H., Hamaguchi, M., & Saito, H. (1997). Constitutive activation of mitogen-activated protein kinase pathway in acute leukemia cells. *Leukemia*, *11*, 479–484.

Treiber, D. K., & Shah, N. P. (2013). Ins and Outs of Kinase DFG Motifs. *Chemistry & Biology*, *20*, 806–815.

Trendowski, M., Wong, V., Yu, G., & Fondy, T. (2015). Enlargement and Multinucleation of U937 Leukemia and MCF7 Breast Carcinoma Cells by Antineoplastic Agents to Enhance Sensitivity to Low Frequency Ultrasound and to DNA-directed Anticancer Agents. *Anticancer Research*, *35*, 65–76.

Trifa, A. P., Popp, R. A., Cucianu, A., Coada, C. A., Urian, L. G., Miltaru, M. S., … Pop, I. V. (2012). Absence of BRAF V600E mutation in a cohort of 402 patients with various chronic and acute myeloid neoplasms. *Leukemia & Lymphoma*, *53*, 2496–2497.

Uings, I. J., & Farrow, S. N. (2000). Cell receptors and cell signalling. *Molecular Pathology*, *53*, 295–299. Underwood, D. C., Osborn, R. R., Kotzer, C. J., Adams, J. L., Lee, J. C., Webb, E. F., … Griswold, D. E. (2000). SB 239063, a Potent p38 MAP Kinase Inhibitor, Reduces Inflammatory Cytokine Production, Airways Eosinophil Infiltration, and Persistence. *The Journal of Pharmacology and Experimental Therapeutics*, *293*, 281–288.

Van Dam, H., Wilhelm, D., Herr, I., Steffen, A., Herrlich, P., & Angel, P. (1995). ATF-2 is preferentially activated by stress-activated protein kinases to mediate c-jun induction in response to genotoxic agents. *The EMBO Journal*, *14*, 1798–1811.

van den Blink, B., Branger, J., Weijer, S., van Deventer, S. J. H., van der Poll, T., & Peppelenbosch, M. P. (2001). Human Endotoxemia Activates p38 MAP Kinase and p42/44 MAP Kinase, But Not c-Jun N-terminal Kinase. *Molecular Medicine*, *7*, 755–760.

Vinay, D. S., Ryan, E. P., Pawelec, G., Talib, W. H., Stagg, J., Elkord, E., … Kwon, B. S. (2015). Immune evasion in cancer: Mechanistic basis and therapeutic strategies. *Seminars in Cancer Biology*, *35*, 185–198.

Vogelstein, B., Civin, C. I., Preisinger, A. C., Krischer, J. P., Steuber, P., Ravindranath, Y., … Bos, J. (1990). RAS gene mutations in childhood acute myeloid leukamia: A pediatric oncology group study. *Genes, Chromosomes and Cancer*, *2*, 159–162.

Volloch, V., Mosser, D. D., Massie, B., & Sherman, M. Y. (1998). Reduced thermotolerance in aged cells results from a loss of an hsp-72 mediated control of JNK signalling pathway. *Cell Stress & Chaperones*, *3*, 265–271.

Vrana, J. A., & Grant, S. (2001). Synergistic induction of apoptosis in human leukemia cells (U937) exposed to bryostatin 1 and the proteasome inhibitor lactacystin involves dysregulation of the PKC/MAPK cascade. *Blood*, *97*, 2105–2114.

Vuci, D., Dixit, V. M., & Wertz, I. E. (2011). Ubiquitylation in apoptosis: a post-translational modification at the edge of life and death. *Nature Reviews Molecular Cell Biology*, *12*, 439–452.

Wada, T., & Penninger, J. M. (2004). Mitogen-activated protein kinases in apoptosis regulation. *Oncogene, 23*, 2838-2849.

Wagner, E. F., & Nebreda, Á. R. (2009). Signal integration by JNK and p38 MAPK pathways in cancer development. *Nature Reviews*, *9, 537-549*.

Walsh, J. G., Cullen, S. P., Sheridan, C., Uthi, A. U., Gerner, C., & Martin, S. J. (2008). Executioner caspase-3 and caspase-7 are functionally distinct proteases. *PNAS*, *105*, 12815–12189.

Wang, J. H., Zhou, Y. J., He, P., & Chen, B. Y. (2007). Roles of mitogen-activated protein kinase pathways during Escherichia coli-induced apoptosis in U937 cells. *Apoptosis, 12,* 375-385.

Wang, J., Tang, R., Lv, M., Wang, Q., Zhang, X., Guo, Y., … Zhang, J. (2011). Defective anchoring of JNK1 in the cytoplasm by MKK7 in Jurkat cells is associated with resistance to Fas-mediated apoptosis. *Molecular Biology of the Cell*, *22*, 117–127.

Wang, T.-H., Wang, H.-S., Ichijo, H., Giannakakouţ, P., Foster, J. S., Fojoţ, T., & Wimalasena, J. (1998). Microtubule-interfering Agents Activate c-Jun N-terminal Kinase/Stress-activated Protein Kinase through Both Ras and Apoptosis Signal-regulating Kinase Pathways. *The Journal of Biological Chemistry*, *273*, 4928–4936.

Wellbrock, C., Karasarides, M., & Marais, R. (2004). The Raf proteins take centre stage. *Nature Reviews Molecular Cell Biology*, *5*, 876–886.

Westwick, J. K., Weitzel, C., Minden, A., Karin, M., & Brenner, D. A. (1994). Tumor necrosis factor α stimulates AP-1 activity through prolonged activation of the c-Jun kinase. *Journal of Biological Chemistry*, *269*, 26396–26401.

Widmann, C., Gerwins, P., Johnson, N. L., Jarpe, M. B., & Johnson, G. L. (1998). MEK Kinase 1, a Substrate for DEVD-Directed Caspases, Is Involved in Genotoxin-Induced Apoptosis. *Molecular and Cellular Biology* , *18*, 2416–2429.

Willoughby, E. A., Perkins, G. R., Collins, M. K., & Whitmarsh, A. J. (2003). The JNK-interacting protein-1 scaffold protein targets MAPK phosphatase-7 to dephosphorylate JNK. *The Journal of Biological Chemistry*, *278*, 10731–10736.

Woessmann, W., & Meng, Y. H. (1999). An essential role for mitogen-activated protein kinases, ERKs, in preventing heat-induced cell death. *Journal of*

Cellular Biochemistry, *15*, 648–662.

Woods, D., & Turhci, J. J. (2013). Chemotherapy induced DNA damage response. Convergence of drugs and pathways. *Cancer Biology & Therapy*, *14*, 379–389.

Wortzel, I., & Seger, R. (2011). The ERK Cascade: Distinct Functions within Various Subcellular Organelles. *Genes & Cancer*, *2*, 195–200.

Wu, W., Alexis, N. E., Bromberg, P. A., Jaspers, I., & Peden, D. B. (2009). Mechanisms of LPS-induced CD40 expression in human peripheral blood monocytic cells. *Biochemical and Biophysical Research Communications, 379,* 573-577.

Xie, Y., Peng, Z., Shi, M., Ji, M., Guo, H., & Shi, H. (2014). Metformin combined with p38 MAPK inhibitor improves cisplatin sensitivity in cisplatin‐resistant ovarian cancer. *Molecular Medicine Reports*, 2346–2350.

Xu, X., Heidenreich, O., Kitajima, I., McGuire, K., Li, W., Su, B., & Nerenberg, M. (1996). Constitutively activated JNK is associated with HTLV-1 mediated tumorigenesis. *Oncogene*, *13*, 135–142.

Yanagisawa, K., Yamauchi, H., Kaneko, M., Kohno, H., Hasegawa, H., & Fujita, S. (1998). Suppression of Cell Proliferation and the Expression of a bcr-abl Fusion Gene and Apoptotic Cell Death in a New Human Chronic Myelogenous Leukemia Cell Line, KT-1, by Interferon-α. *Blood*, *191*, 641–648.

Yang, F., Teves, S. S., Kemp, C. J., & Henikoff, S. (2014). Doxorubicin, DNA torsion, and chromatin dynamics. *Biochimica et Biophysica Acta, 1845,* 84-89*.*

Yang, H., Higgins, B., Kolinsky, K., Packman, K., Go, Z., Iyer, R., … Su, F. (2010). RG7204 (PLX4032), a selective BRAFV600E inhibitor, displays potent antitumor activity in preclinical melanoma models. *Cancer Research*, *70*, 5518–5527.

Yang, S. H., Whitmarsh, A. J., Davis, R. J., & Sharrocks, A. D. (1998). Differential targeting of MAP kinases to the ETS-domain transcription factor Elk-1. *The EMBO Journal* , *17*, 174–1749.

Yang, Y., Huang, Q., Lu, Y., Li, X., & Huang, S. (2012). Reactivating PP2A by FTY720 as a novel therapy for AML with C-KIT tyrosine kinase domain mutation. *Journal of Cellular Biochemistry, 113,* 1314-1322.

Yarza, R., Vela, S., Solas, M., & Ramirez, M. J. (2015). c-Jun N-terminal Kinase (JNK) Signaling as a Therapeutic Target for Alzheimer's Disease. *Frontiers in Pharmacology*, *6*, 1–12.

Yeh, T. C., Marsh, V., Bernat, B. A., Ballard, J., Colwell, H., Evans, R. J., … Wallace, E. (2007). Biological characterization of ARRY-142886 (AZD6244), a potent, highly selective mitogen-activated protein kinase kinase 1/2 inhibitor. *Clinical Cancer Research*, *13*, 1576–1583.

Yoon, S., & Seger, R. (2006). The extracellular signal-regulated kinase: Multiple substrates regulate diverse cellular functions. *Growth Factors*, *25*, 21–44.

Yoshida, K., Nagai, T., Ohmine, K., Uesawa, M., Sripayap, P., Ishida, Y., & Ozawa, K. (2011). Vincristine potentiates the anti-proliferative effect of an aurora kinase inhibitor, VE-465, in myeloid leukemia cells. *Biochemical Pharmacology*, *82*, 1884–1890.

Young, P. R., McLaughlin, M. M., Kumar, S., Kassis, S., Doyle, M. L., McNulty, D., … Lee, J. C. (1997). Pyridinyl imidazole inhibitors of p38 mitogen-activated protein kinase bind in the ATP site. *Journal of Biological Chemistry, 272,* 12116-12121.

Yu, C., Krystal, G., Varticovksi, L., Mckinstry, R., Rahmani, M., Dent, P., & Grant, S. (2002). Pharmacologic Mitogen-activated Protein/Extracellular Signal-regulated Kinase Kinase/Mitogen-activated Protein Kinase Inhibitors Interact Synergistically with STI571 to Induce Apoptosis in Bcr/Abl-expressing Human Leukemia Cells. *Cancer Research*, *62*, 188–199.

Zarubin, T., & Han, J. (2005). Activation and signaling of the p38 MAP kinase pathway. *Cell Research*, *15*, 11–18.

Zhang, S., Han, J., Sellsţ, M. A., Chernofftţ, J., Knaus, U. G., Ulevitch, R. J., & Bokoch, G. M. (1995). Rho Family GTPases Regulate p38 Mitogen-activated Protein Kinase through the Downstream Mediator Pak1. *Journal of Biological Chemistry*, *270*, 23934–23936.

Zhang, T., Inesta-vaquera, F., Niepel, M., Zhang, J., Scott, B., Machleidt, T., … Gray, N. S. (2012). Discovery of potent and selective covalent inhibitors of JNK. *Chemical Biology*, *19*, 140–154.

Zhang, W., Konopleva, M., Shi, Y. X., McQueen, T., Harris, D., Ling, X., … Andreeff, M. (2008). Mutant FLT3: A direct target of sorafenib in acute myelogenous leukemia. *Journal of the National Cancer Institute, 100,* 184-198.

Zhang, W., & Liu, H. (2002). MAPK signal pathways in the regulation of cell proliferation in mammalian cells. *Cell Research*, *12*, 9–18.

Zhang, Y., & Dong, C. (2005). MAP Kinases in Immune Responses. *Cellular & Molecular Immunology Review*, *2*, 20-27.

Zhao, X., & Guan, J.-L. (2011). Focal adhesion kinase and its signaling pathways in cell migration and angiogenesis. *Advanced Drug Delivery Reviews*, *63*, 610–615.

Zhou, C., Chen, Z., Lu, X., Wu, H., Yang, Q., & Xu, D. (2016). Icaritin activates JNK-dependent mPTP necrosis pathway in colorectal cancer cells. *Tumor Biology*, *37*, 3135–3144.

Zhou, J., An, H., Xu, H., Liu, S., & Cao, X. (2005). Heat shock up-regulates expression of Toll-like receptor-2 and Toll-like receptor-4 in human

monocytes via p38 kinase signal pathway. *Immunology, 114,* 522-530.

Zhou, Z., Lu, X., Wang, J., Xiao, J., Liu, J., & Xing, F. (2016). microRNA let-7c is essential for the anisomycin-elicited apoptosis in Jurkat T cells by linking JNK1/2 to AP-1/STAT1/STAT3 signaling. *Nature*, *6*, 1-13.

Ziegler, U. (2004). Morphological Features of Cell Death. *News in Physiological Sciences*, *19*, 124–128.

Ziyad, S., & Iruela-Arispe, M. L. (2011). Molecular Mechanisms of Tumor Angiogenesis. *Genes & Cancer*, *2*, 1085–1095.

Zou, H., Li, Q., Lin, S. C., Wu, Z., Han, J., & Ye, Z. (2007). Differential requirement of MKK4 and MKK7 in JNK activation by distinct scaffold proteins. *FEBS Letters, 581,* 196-202.

Zou, X., & Blank, M. (2017). Targeting p38 MAP kinase signaling in cancer through post-translational modifications. *Cancer Letters*, *384*, 19–26.

Zou, X., & Calame, K. (1999). Signaling pathways activated by oncogenic forms of Abl tyrosine kinase. *Journal of Biological Chemistry, 274,* 18141-18144.